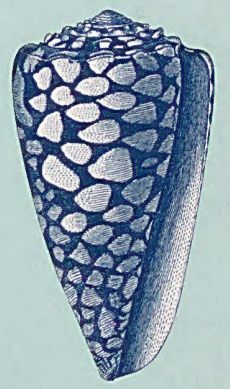

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Embryonic and larval development of *Pinctada margaritifera* (Linnaeus, 1758)

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Abstract

We describe the developmental stages of the black-lip pearl oyster, *Pinctada margaritifera* (Linnaeus, 1758), larvae from fertilisation through embryonic development and larval growth in the laboratory at 28 ± 1°C. Larvae were anesthetised, fixed, critical-point dried and examined using a scanning electron microscope. We examined embryonic development (fertilisation, polar body, blastomeres, gastrula) and attributes of the larval shell (size, prodissoconch I/II, growth lines, provinculum, shell fracture) and larval velum. The first polar body formed 24 min after fertilisation and fertilised eggs had a mean diameter of 59.9 ± 1.4 µm. The earliest actively swimming trochophore appeared 8–12 h after fertilisation. The D stage was reached approximately 24 h after fertilisation and measured 79.7 ± 2.3 µm in shell length. Ten-day-old larvae had umbones that arose opposite each other above the hinge axis and 22-day-old larvae, with a mean shell length of 230.8 ± 4.9 µm, developed a pigment spot just before entering the pediveliger stage.

Additional keywords: hinge, morphology, pearl oyster, scanning electron microscope.

Introduction

Herdman (1903) studied the early life stages of *Pinctada vulgaris* (= *P. fucata* Gould, 1850) larvae up to 3 days after fertilisation. Other studies have investigated larval development and growth of *P. fucata*, *P. martensi* Dunker, 1850 and *P. maxima* Jameson, 1901 (Ota 1957; Minaur 1969; Tanaka and Kumeta 1981; Alagarswami *et al.* 1983). Rose and Baker (1994) described larval development of *P. maxima* in detail and compared their findings with those reported for other pearl oyster species. Although *P. margaritifera* larvae have been cultured since 1970 (Tanaka *et al.* 1970; Alagarswami *et al.* 1989; Southgate and Beer 1997), embryonic development and the morphology of different larval stages have not been described in detail.

The present study notes the characteristics of *P. margaritifera* larvae that have not been reported on previously using scanning electron microscopy (SEM) and provides a basic understanding of larval development during hatchery culture.

Materials and methods

Larval rearing

Pinctada margaritifera broodstock were induced to spawn by thermal stimulation and the addition of sperm in a seawater suspension. Fertilised eggs were stocked at a density of 30 mL^{-1} in aerated fibreglass tanks (500 L) filled with 1 µm filtered seawater at 28°C. The salinity of seawater was 33, which was measured using the practical salinity scale. After 24 h, when D-stage larvae (shell becomes D-shaped) had a mean shell length of 79.7 ± 2.3 µm, they were collected on a 25-µm mesh sieve, counted and placed at a density of 2 mL^{-1} in 500-L aerated fibreglass tanks containing filtered seawater at the same temperature and salinity.

We cultured Tahitian *Isochrysis* aff. *galbana* Green (T-ISO) and *Pavlova salina* Green in 3-L glass flasks and 20-L carboys in autoclaved 0.45-µm-filtered and ultraviolet (UV)-treated seawater using f/2 nutrient

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medium (Guillard 1983). Microalgae cultures were provided with illumination from cool white fluorescent lights with a 12-h light:12-h dark photoperiod. Larvae were fed daily a 1:1 mixture of T-ISO and *Pavlova salina* at a ration of $1–18 \times 10^3$ cells mL^{-1} (Southgate and Beer 1997; Doroudi *et al.* 1999*a*). We conducted three separate spawnings to collect embryonic and larval samples.

Sample preparation

We observed embryonic development every 15 min during the first 3 h, then once an hour until the trochophore stage (8–24 h) and D-stage (24 h) using a compound microscope. Larvae were narcotised in a 15% (w/v) solution of $MgCl_2$ (Bellolio *et al.* 1993) and seawater (1:1) at 28°C for 5–10 min. We collected samples from three tanks at 2-day intervals after the D stage (24 h) and fixed them in 2.5% glutaraldehyde in 0.1 M piperazine at pH 7.6. This sampling continued until larvae had developed to the 'eyed' stage (i.e. when larvae develop a pigment spot). Subsamples of larvae were post-fixed with osmium tetroxide (OsO_4), dehydrated in a graded series of ethanol, critical-point dried in liquid carbon dioxide (CO_2), mounted on aluminium stubs with double-sided tape and coated with gold before being examined using an SEM.

We collected larvae on a mesh sieve, washed them into a graduated cylinder and removed a subsample, from which the shell length of 40 larvae was measured using a compound microscope. Morphological terminology follows that commonly used for bivalve larvae in similar studies (Waller 1981; Bellolio *et al.* 1993).

Results

The time series of developmental stages of *P. margaritifera* embryos and larvae is shown in Table 1.

Embryo to trochophore

Unfertilised *P. margaritifera* eggs had a mean diameter of 39.7 ± 1.3 µm ($n = 40$; Fig. 1). The first polar body formed 24 min after fertilisation and fertilised eggs had a mean diameter of 59.9 ± 1.4 µm ($n = 40$). The four blastomeres resulting from the second cleavage formed 2 h after fertilisation. Cell division followed the usual bivalve pattern for bivalves and resulted in the gastrula, 5 h after fertilisation. The change from a ciliated gastrula to the trochophore stage was gradual and the earliest actively swimming trochophore appeared 8–12 h after fertilisation. Morphological changes from trochophore to the D stage included extension along the longitudinal axis and the apical region becoming broader than the posterior region. At this time, cilia on the apical region became longer. With the development of long cilia, larvae began to secrete shell and the resulting larvae swam actively using the velum.

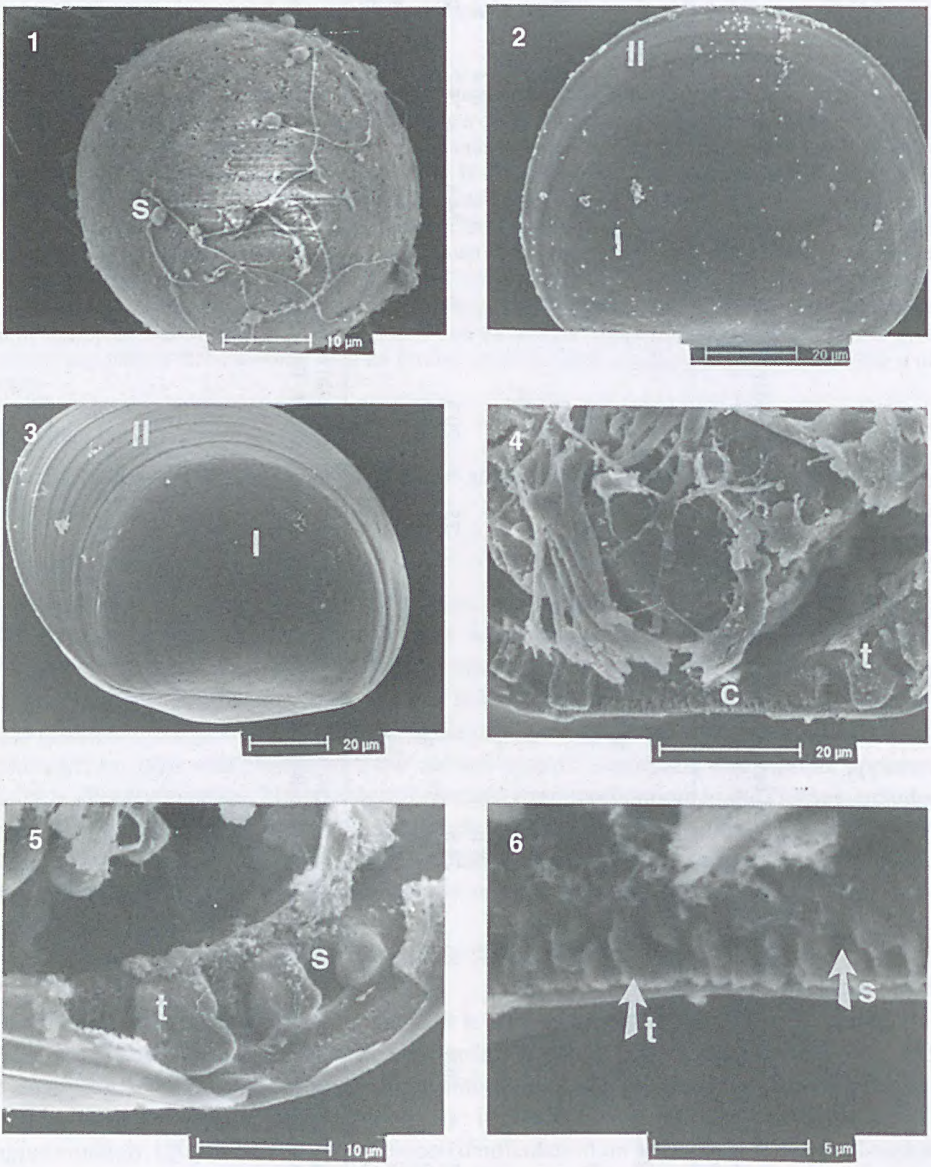
Larvae

The D stage was reached approximately 24 h after fertilisation and larvae measured 79.7 ± 2.3 µm ($n = 40$) in shell length. The D-stage larvae showed preliminary growth rings after 2 days (Fig. 2). The shell showed slight umbonal growth after 6 days development and prodissoconch I and II could be clearly identified (Fig. 3). At a shell length of approximately 100 µm, the hinge developed denticulation on either side of a central region. As the larva grew, the hinge developed and formed a series of teeth and sockets on each valve (Fig. 4). Each valve had teeth on either side of a central area (Fig. 5). The central area included a series of tiny teeth and sockets (Fig. 6). Ten-day-old larvae had umbones that arose opposite each other above the hinge axis (Fig. 7) and 22-day-old larvae, with a mean shell length of 230.8 ± 4.9 µm ($n = 40$), developed a pigment spot and entered the pediveliger stage shortly after (Fig. 8). Sections of broken shell edges at this stage suggest that calcification of the shell by the mantle had occurred in prodissoconch II (Fig. 9). Fractures through prodissoconch II showed layering in the shell structure and indicated that the shell is thicker in the area of growth lines (Fig. 10). The oval velum was located at the

Table 1. Embryo and larval development of some important pearl oyster species

	Pinctada fucata		*Pinctada maxima*						*Pinctada margaritifera*			
Authors*	1		2		3		4		5		6	
Temperature (°C)	24.3–29.8		25.0–29.5		27–31		26–28		26–30		27–29	
Stage	S	T	S	T	S	T	S	T	S	T	S	T
Egg	47	–	–	–	59	–	–	–	45	–	49.7	–
Polar body	–	–	–	–	–	–	–	–	–	–	59.9	24 min
Four blastomere	–	–	60	1 h	–	–	–	–	–	–	60	2 h
Gastrula	–	–	75	5 h	–	–	–	–	–	–	69.6	5 h
Trochophore	–	–	75	8 h	–	–	–	–	–	–	70.4	8–12 h
D shape	67	20 h	85	24 h	–	–	75	20 h	75	24 h	79.7	24 h
Early umbo	100	–	110	8 d	96	5 d	115	10 d	110	9 d	109.9	8 d
Umbo	135	11 d	140	14 d	125	12 d	–	–	140	12 d	140.7	12 d
Eye-spot	210	15 d	235	21 d	–	–	–	–	210	16 d	230.8	22 d

Mean size (S) of either the egg or larval diameter or shell length (in µm); time (T) is given in minutes (min), hours (h) or days (d).
*The sources of the data are as follows: 1, Alagarswami *et al.* (1983); 2, Rose and Baker (1994); 3, Minaur (1969); 4, Tanaka and Kumeta (1981); 5, Alagarswami *et al.* (1989); 6, present.study.



Figs 1–6. *1*, Unfertilised egg of *Pinctada margaritifera* with sperm (s) on the surface. *2*, External view of the right valve of *Pinctada margaritifera* D-stage larvae; I, prodissoconch I; II, prodissoconch II. *3*, Prodissoconch I (I) and II (II) in the early umbo stage of *Pinctada margaritifera* larvae. *4*, Development of the provinculum of *Pinctada margaritifera* larvae (umbo stage); t, tooth; c, central area. *5*, Main teeth on either side of a central area of *Pinctada margaritifera* larvae (umbo stage); t, tooth; s, socket. *6*, Central area of the *Pinctada margaritifera* larval hinge (umbo stage) showing the series of teeth (t) and sockets (s).

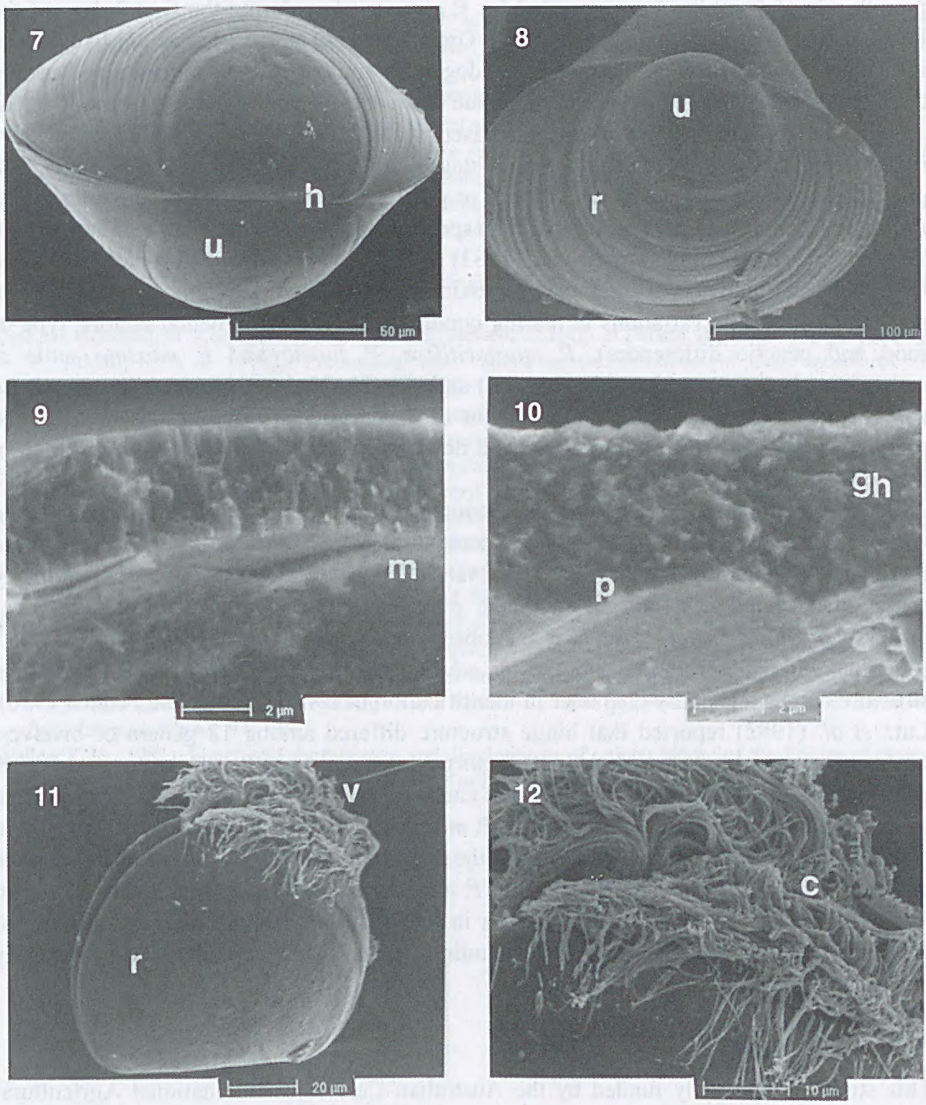


Fig. 7–12. *7*, Hinge length (h) and umbones (u) arise over the hinge axis of *Pinctada margaritifera* larvae (umbo stage). *8*, An eyed larva of *Pinctada margaritifera* showing details of umbonal features (u); r, right valve. *9*, The mantle (m) viewed from a cross-section of the prodissoconch II (II) of *Pinctada margaritifera* larvae (umbo stage). *10*, Fracture of the prodissoconch in *Pinctada margaritifera* umbo larva; p, outer prismatic layer; gh, granular homogeneous layer. *11*, Lateral view of the entire *Pinctada margaritifera* D-stage larva with velum (v) extended at the anterior dorsal side of the shell; r, right valve. *12*, Enlargement of cilia (c) on the velum of *Pinctada margaritifera* D-stage larvae.

posterior dorsal side of the larva (Fig. 11) and was well developed with a peripheral ring of cilia (Fig. 12).

Discussion

Pinctada margaritifera larvae need a period of 8 days to reach the early umbo stage (shell length 110 µm) and exhibit an average daily growth rate of 3.7 µm. Elsewhere, a daily

growth rate of 5 μm has been reported for *P. margaritifera* during the first 7 days of the larval rearing period (Tanaka *et al.* 1970). Growth rates of bivalve larvae are likely to be influenced by genetic factors, as well as endogenous and exdogenous nutrition and culture conditions. Because *P. margaritifera* larvae have exponential growth (Doroudi *et al.* 1999*b*), the mean daily growth rate increased up to 7.2 μm over the period of 22 days required for larvae to reach the eye spot stage (230 μm). Eye spots in *P. margaritifera* generally occur in larvae that are 230 μm or greater in shell length. In a previous study, *P. margaritifera* larvae developed a pigment spot at 210 μm shell length (Alagarswami *et al.* 1989). In *P. fucata* (Alagarswami *et al.* 1983) and *P. maxima* (Rose and Baker 1994), eye spots form in individuals that are approximately 210 and 230 μm in shell length, respectively. Despite variations in rearing conditions (e.g. environmental factors, type of food and genetic differences), *P. margaritifera*, *P. fucata* and *P. maxima* settle at approximately the same size (230–266 μm) and age (20–23 days) from fertilisation (Rose and Baker 1994). The overall development of *P. margaritifera* larvae described in the present study is similar to the more general descriptions of pearl oyster larvae reported in previous studies (Table 1).

The larvae of bivalves are similar in exterior appearance and are difficult to differentiate without detailed anatomical study. The present SEM study of *P. margaritifera* larvae revealed some anatomical features of the larval shell that have not been observed previously using other techniques. For instance, the punctate region on the exterior surface of prodissoconch I of *P. margaritifera* is also observed in *Crassostrea virginica* Gmelin, 1791 (Carriker and Palmer 1979) and *Ostrea edulis* Linnaeus, 1750 (Waller 1981). Hinge structure can be a primary character in identification of bivalve larvae (Le Pennec 1980). Lutz *et al.* (1982) reported that hinge structure differed among 12 genera of bivalves, whereas the basic hinge morphology of *P. margaritifera* seems to be similar to that of other pearl oysters; that is, a tooth and socket at each end with a thin central area. The present study has shown that 8-day-old larvae of *P. margaritifera*, with a shell length of 110 μm, have five teeth in each valve, with three at the anterior end of the hinge line and two at the posterior. This is the same as reported for *P. maxima* larvae with a shell length of 90 μm (Rose and Baker 1994). Our observations in the present study on the larval rearing of *P. margaritifera* provide a basic understanding of larval development during hatchery culture of this species.

Acknowledgments

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Ultrastructure of male germ cells in the testes of abalone, *Haliotis ovina* Gmelin

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Abstract

An ultrastructural study of male germ cells in the testes of *Haliotis ovina* revealed that spermatogenesis could be classified into 13 stages, based on the pattern of chromatin condensation and distribution of organelles, as follows: the spermatogonium; five stages of the primary spermatocyte; the secondary spermatocyte; five stages of the spermatid; and the spermatozoa. Each spermatogonium was round or oval, with a euchromatic nucleus and prominent nucleolus. The primary spermatocytes were divided into five stages: leptotene (LSc); zygotene (ZSc); pachytene (PSc); diplotene (DSc); and metaphase (MSc). The nucleus of the LSc contained scattered small heterochromatin blocks that were increasingly thickened in the ZSc. The PSc was characterised by a bouquet pattern of heterochromatin fibres. The DSc decreased in size, resulting in close clumping of chromatin blocks, whereas in the MSc, long and large blocks of chromosomes were formed and then moved to be aligned along the equatorial region. Secondary spermatocyte showed thick chromatin blocks that appeared reticulate. The spermatid could be divided into five stages (St_{1-5}). The St_1 was a large round cell and its nucleus contained homogeneous chromatin granules. In St_2, the nuclear chromatin started to condense into patches. The St_3 was smaller with a round nucleus containing dark blocks of heterochromatin. The St_4 became smaller still, with a round opaque nucleus. The St_5 was the smallest round cell, with almost completely condensed chromatin. The spermatozoon had a round to barrel-shaped head that contained completely condensed chromatin covered by a conical acrosome. The posterior border of the nucleus was flanked by five large spherical mitochondria and the tail consisted of axonemal microtubules surrounded by the plasma membrane.

Introduction

There are almost 100 species of abalone all belonging to the genus *Haliotis* (Fallu, 1991). These snails are widely distributed in tropical and temperate seas and inhabit submerged rock (Crofts 1929). Since ancient times, the abalone has been an economically important snail because of its decorative shell and food value. Abalone are commercially important molluscs in many countries, such as Japan, America, Mexico and Australia, where the culture of commercial abalone is well established. In Thailand, there are three species of abalone, namely *Haliotis ovina* Gmelin, 1791, *H. asinina* Linnaeus, 1758 and *H. varia* Linnaeus, 1929 (Nateewathana and Hylleberge 1986; Tookvinart *et al.* 1986; Bussarawit *et al.* 1990). Of these the three species, *H. ovina* is the one with economic potential (Nateewathana and Hylleberge 1986; Bussarawit *et al.* 1990).

Species of *Haliotis* are dioecious, with clear sexual dimorphism. The single testis envelops the digestive gland and, together, these structures form a large cone-shaped appendage called the conical organ, which wraps around the right and posterior margins of the shell muscle (Crofts 1929). The colour of the gonad indicates the sex of the abalone. The testis is cream to ivory, whereas the ovary is green in colour (Singhagraiwan 1989).

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There is no genital duct in *Haliotis* (Crofts 1929). The sperm from the testis are expelled into the cavity of the right renal organ, which is seen on the dorsal surface of the body and is overlapped by the digestive gland, and finally pass through shell perforations, which are located on the left side (Fallu 1991), into the water.

There have been extensive ultrastructural studies of male germ cells, especially the spermatozoa, in several haliotid species, such as *Haliotis rufescens* Swainson, 1822 (Lewis *et al.* 1980), *H. aquatilis* Reeve, 1846 (Shiroya and Sakai 1992), *H. diversicolor supertexta* Lischke, 1870 (Gwo *et al.* 1997), *H. discus* Reeve, 1846 (Sakai *et al.* 1982; Usui 1987), *H. midae* Linnaeus, 1758 (Hodgson and Foster 1992), *H. laevigata* Donovan, 1808 (Healy *et al.* 1998), and *H. asinina* (Apisawetakan *et al.* 2000; Sobhon *et al.* 2001). In general, spermatogenesis in these haliotid species can be classified into the following stages: spermatogonia; primary spermatocytes; secondary spermatocytes; spermatids; and spermatozoa. Sobhon *et al.* (2001) differentiated male germ cells in *H. asinina* into 14 stages based on the ultrastructure and pattern of chromatin condensation. These stages were the spermatogonium, six stages of the primary spermatocyte, the secondary spermatocyte, four stages of the spermatid and the spermatozoa (immature and mature). The general ultrastructure of haliotid spermatozoa is typical of the primitive type described for molluscs that reproduce by external fertilisation. The spermatozoa have a very simplified midpiece that is composed of a cluster of spherical mitochondria surrounding a pair of orthogonally arranged centrioles (Lewis *et al.* 1980; Sakai *et al.* 1982; Hodgson and Bernard 1986; Hodgson and Foster 1992; Shiroya and Sakai 1992; Gwo *et al.* 1997; Healy *et al.* 1998; Apisawetakan *et al.* 2000).

In the present study, we examined the ultrastructure of male germ cells in *H. ovina*, an abalone of potential economic importance, which is common along the coast of Thailand. The results are compared with those of *H. asinina* and other abalone species.

Materials and methods

Adult *H. ovina* (approximately 7 cm shell length, 170 g weight) were collected during June and July 1999 from Samed Island, Rayong Province, Thailand. A total of 20 male abalone was used in the present study. For the ultrastructural study, very small pieces of testes were fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.8) at 4°C overnight, washed in 0.1 M sodium cacodylate buffer and post-fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 h at 4°C. The pieces were then dehydrated in a graded series of ethanol, cleared using propylene oxide and infiltrated and embedded in Araldite 520 epoxy resin. Sections were cut with glass knives on a Sorvall MT-2 ultramicrotome. Semithin sections were stained with toluidine blue and observed with a light microscope. Ultrathin sections were stained with uranyl acetate and lead citrate and were viewed with a Hitachi H300 transmission electron microscope (TEM) operated at 75 kV.

Results

The male germ cells of *H. ovina* can be classified into 13 stages based on cell shape and size, nuclear shape and size, and pattern of chromatin condensation (Fig. 1). These stages comprise the spermatogonium, five stages of the spermatocyte, the secondary spermatocyte, five stages of the spermatid and the spermatozoa.

Spermatogonium

These cells lie on the outer edges of the lobe of the testis. The spermatogonium is spherical or oval, with a diameter of approximately 8–10 μm (Fig. 1*A*,*B*). The nucleus is round or slightly oval and contains mostly euchromatin, with only a thin rim of heterochromatin blocks attached to the inner surface of the nuclear envelope (Fig. 2*A*). The nucleolus is

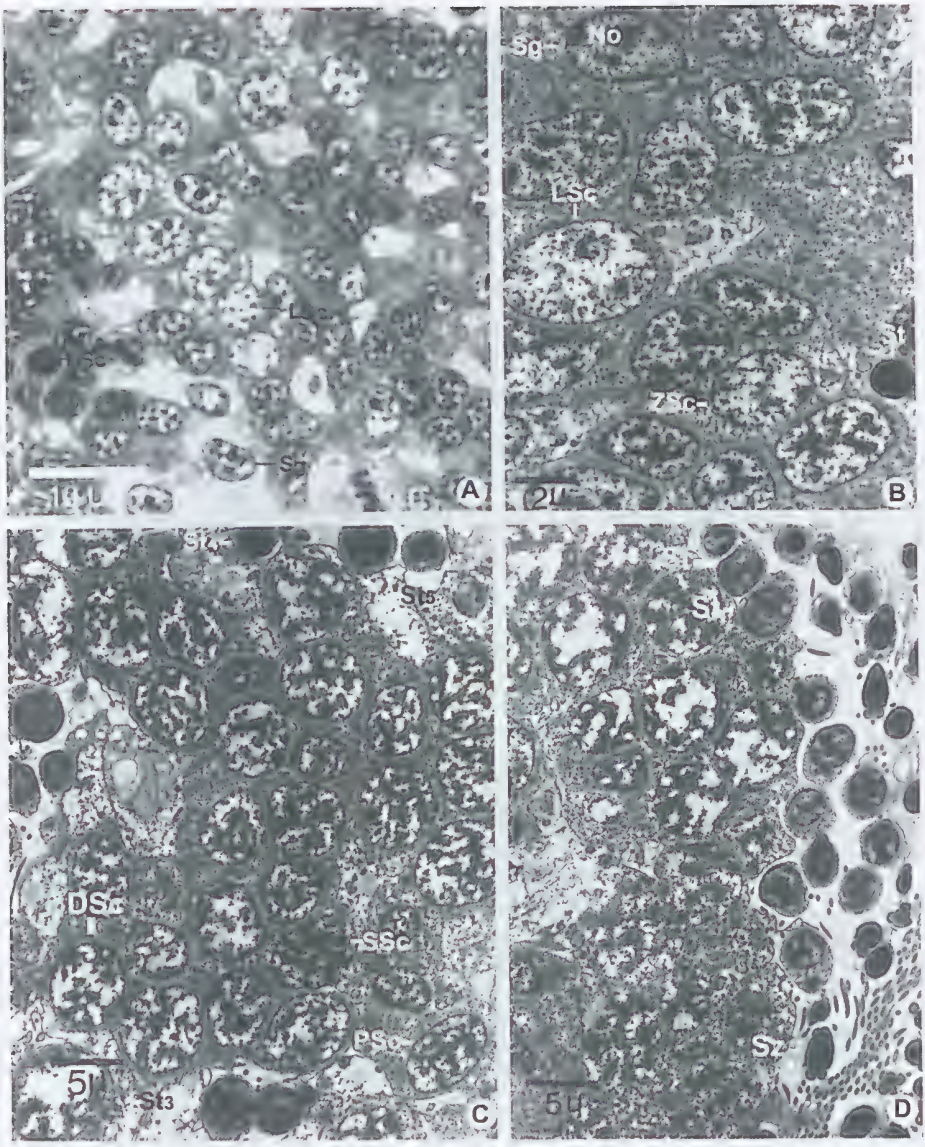

Fig. 1. Photomicrograph (*A*) and electron micrographs (*B–D*) showing various stages of the male germ cells of *Haliotis ovina*. Sg, Spermatogonia; LSc, leptotene primary spermatocyte; ZSc, zygotene primary spermatocyte; PSc, pachytene primary spermatocyte; DSc, diplotene primary spermatocyte; SSc, secondary spermatocyte; St, spermatid; Sz, spermatozoa.

prominent against the background of a rather transparent nucleoplasm. The cytoplasm contains free ribosomes and numerous mitochondria, which appear at the outer surface of the nuclear envelope (Fig. 2*A*).

Spermatocytes

The primary spermatocytes (PrSc) consist of five stages: leptotene (LSc), zygotene (ZSc), pachytene (PSc), diplotene (DSc) and metaphase (MSc) spermatocytes. The early cells

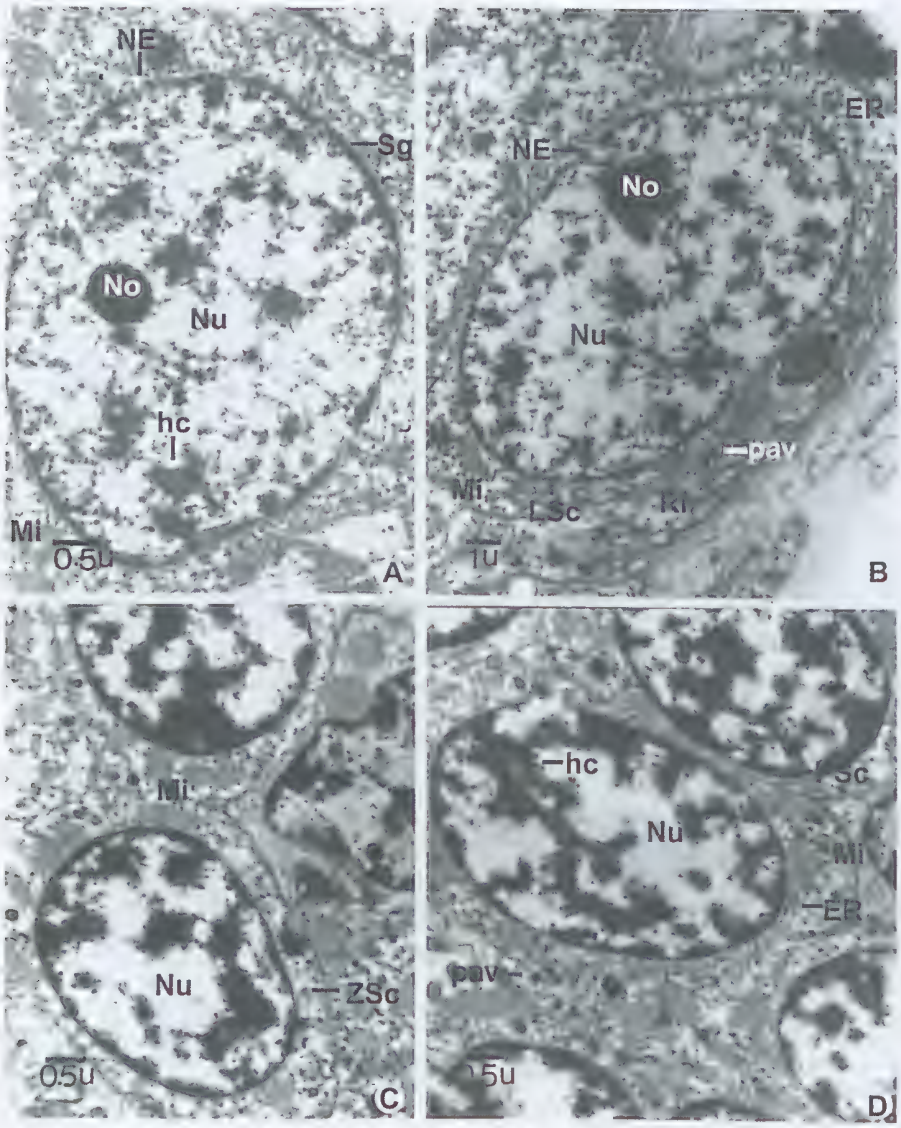

Fig. 2. *A*, High magnification of a spermatogonium (Sg) showing the round nucleus (Nu) with distinct nucleolus (No) and heterochromatin (hc) blocks. Mitochondria (Mi) are numerous on the outer surface of the nuclear envelope (NE). *B*, A leptotene primary spermatocyte (LSc) contains an oval nucleus with nucleolus. The cytoplasm contains mitochondria, ribosomes (Ri), endoplasmic reticulum (ER) and proacrosomal vesicles (pav). *C*, A zygotene primary spermatocyte contains an oval nucleus with dense heterochromatin and mitochondria in the cytoplasm. *D*, A pachytene primary spermatocyte (PSc) contains a nucleus with a bouquet pattern of heterochromatin. Note the presence of ER and mitochondria in the cytoplasm.

(LSc, ZSc, PSc) are round, approximately 12–15 μm in diameter, whereas the late-staged cells (DSc, MSc) are smaller in size (8–10 μm in diameter). Other distinctive differences among various stages of the PrSc are the pattern of chromatin condensation and the relative amount of euchromatin versus heterochromatin.

Leptotene spermatocyte

These cells are round, approximately 12–15 μm in diameter (Fig. 1*A*,*B*). The chromatin, which has started to condense into small blocks of heterochromatin, is scattered evenly throughout the nucleus. The nucleolus is still present, but not as prominent as that of the spermatogonium. The cytoplasm contains a few ribosomes, mitochondria and proacrosomal vesicles (Fig. 2*B*).

Zygotene spermatocyte

These cells are approximately the same size as the LSc (approximately 12 μm in diameter; Fig. 1*B*). Under TEM, the heterochromatin blocks are denser than those of the LSc and the nucleolus disappears (Fig. 2*C*). The cytoplasm contains proacrosomal vesicles and mitochondria, similar to those in the LSc.

Pachytene spermatocyte

These cells are round and their sizes are smaller than the LSc (approximately 10–12 μm in diameter; Fig. 1*C*). The heterochromatin appears as long threads and thick fibres that are entwined into a 'bouquet pattern', and are visible throughout the nucleus (Fig. 2*D*). The cytoplasm contains proacrosomal vesicles; the mitochondria and rough endoplasmic reticulum are greater in number than the LSc (Fig. 2*D*).

Diplotene spermatocyte

These cells are similar to the PSc, but are smaller (approximately 8–10 μm in diameter). The nucleus of the DSc is also smaller than that of the PSc. The chromatin strands, which are distributed among the denser nucleoplasm, become increasingly thicker than those in the earlier stage (Fig. 3*A*). The cytoplasm contains mitochondria and proacrosomal vesicles, similar to those in the PSc.

Metaphase spermatocyte

These cells (approximately 8 μm in diameter) exhibit thick chromosomes that are arranged close together along the equatorial region and the nuclear membrane completely disappears (Fig. 3*B*). The cytoplasm contains mitochondria, numerous ribosomes and proacrosomal vesicles (Fig. 3*B*).

Secondary spermatocyte

These cells are round and smaller than the MSc (approximately 6 μm in diameter; Fig. 1*C*). They are rarely observed and occur after meiosis I. The SSc shows thick chromatin blocks composed of criss-crossing fibres, which appear as reticulate chromatin (Fig. 3*C*). Fewer mitochondria and proacrosomal vesicles are visible in the cytoplasm (Fig. 3*C*).

Spermatids

There are five stages of spermatid (St_{1-5}), differing in size, nuclear shape and chromatin condensation pattern.

Spermatid I

The nuclei of St_1 are round (approximately 6 μm in diameter). The St_1 can be distinguished by their chromatin, which appears as homogeneous granules that are

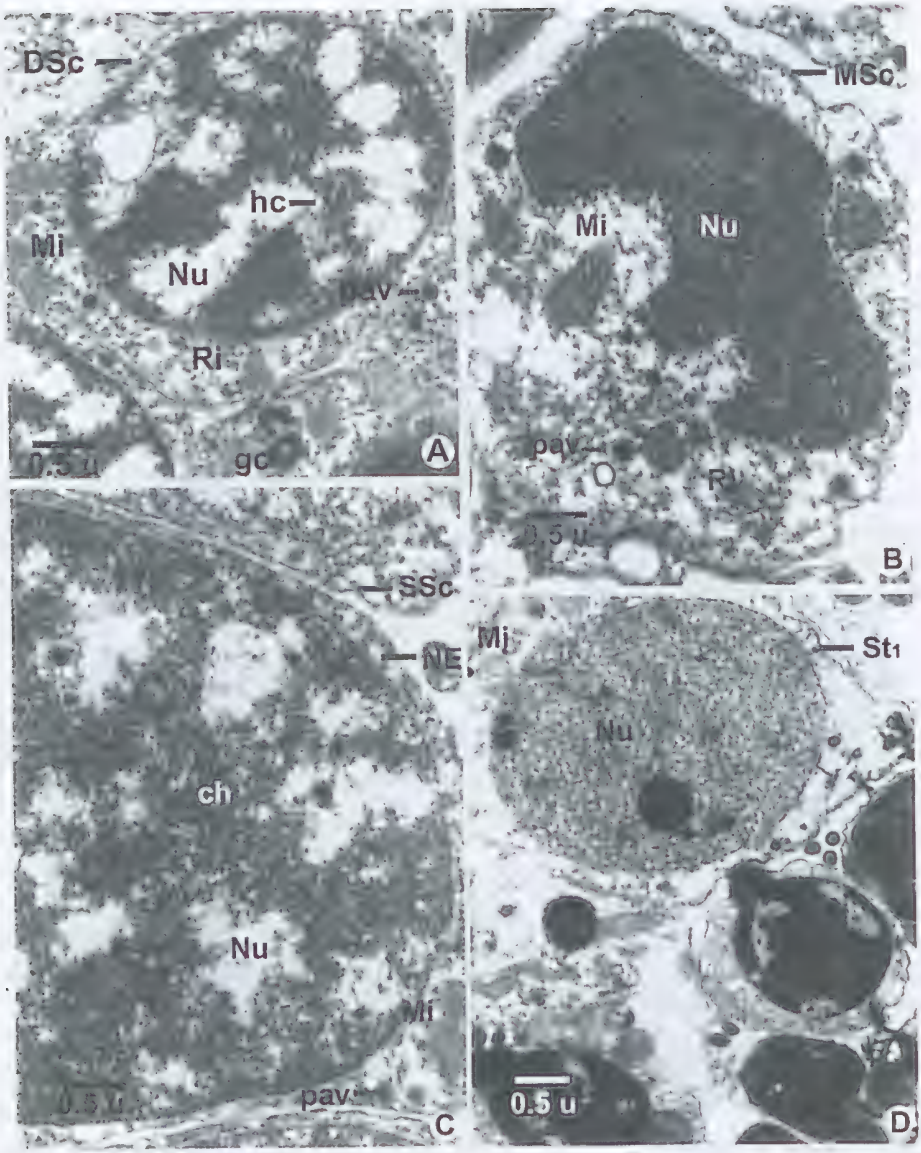

Fig. 3. *A*, A diplotene primary spermatocyte (DSc) contains a round nucleus (Nu) with very thick heterochromatin (hc). Note the presence of mitochondria (Mi), ribosomes (Ri), proacrosomal vesicles (pav) and Golgi body in the cytoplasm. *B*, A metaphase spermatocyte (MSc) exhibits thick chromosomes arranged along the equatorial region. Mitochondria, ribosomes and proacrosomal vesicles are still present. *C*, A secondary spermatocyte (SSc) with a round nucleus showing reticulate chromatin (ch). *D*, Spermatid I (St_1) showing a round nucleus with homogeneous granular chromatin. gc, Golgi complex.

uniformly spaced throughout the nucleus (Fig. 3*D*). As a result, the whole nucleus appears moderately dense without any intervening transparent area of nucleoplasm. The cytoplasm exhibits fewer organelles, such as mitochondria, which tend to be concentrated on one side of the nucleus (Fig. 3*D*).

Spermatid II

The general features of St_2 are similar to those of St_1, but the nucleus, which is still round, is smaller in size in St_2 (approximately 5 μm in diameter) and is located centrally within the cell (Fig. 4*A*). The chromatin appears as homogeneous granules that are uniformly spaced throughout the nucleus and condensed into patches. The cytoplasm contains a few mitochondria on one side and ribosomes (Fig. 4*A*).

Spermatid III

The St_3 is smaller than St_2 (approximately 4 μm in diameter) and the nucleus maintains a round shape. The chromatin begins to condense into dark blocks, with intervening light areas of nucleoplasm (Fig. 4*B*). The cytoplasm contains few ribosomes, mitochondria and proacrosomal vesicles (Fig. 4*B*).

Spermatid IV

The St_4 is smaller (approximately 3 μm in diameter), but still appears round in shape. The chromatin of St_4 is almost completely condensed, resulting in an opaque nucleus (Fig. 4*C*). At this stage, there is a continued loss of cytoplasm, a decrease in nuclear size and condensation of nuclear material. The cytoplasm contains numerous ribosomes, a few large mitochondria and centrioles (Fig. 4*C*).

Spermatid V

The St_5 is the smallest among ther spermiogenic cells (approximately 2 μm in diameter), but is still round in shape. The chromatin of St_5 is almost completely condensed (Fig. 4*D*). Fusion of proacrosomal vesicles into an acrosome that appears slightly indented at the anterior region of the nucleus can be seen (Fig. 4*D*). The cytoplasm contains numerous ribosomes and a few mitochondria (Fig. 4*D*). The mitochondria are fewer, but are larger in size, and occupy the posterior border of the nucleus (Fig. 4*D*).

Spermatozoa

Mature spermatozoa are detached from trabeculae and are arranged in rows further from the former stages, whereas their long tails are pointing outwards and are mingled with those of another spermatogenic unit (Fig. 1*D*). The nucleus (1 × 4 μm in size) is fully elongated and slightly tapered at the anterior end (Fig. 5*A*). The chromatin is completely condensed and the anterior portion of the head is covered by an acrosome that appears as an inverted cup, the concavity of which separates it from the anterior border of the indented nucleus (Fig. 5*A*). This subacrosomal space contains a crystalline acrosomal core embedded in more homogeneous material (Fig. 5*C*). The acrosomal matrix appears homogeneous, with varying degrees of electron opacity (Fig. 5*A*). The nuclear chromatin is completely condensed (Fig. 5*C*). The head of each spermatozoon comprises a barrel-shaped nucleus (Fig. 5*A*). At the posterior border of the nucleus, there are proximal and distal centrioles that are surrounded by a ring of five round mitochondria with cristae (Fig. 5*A*,*B*). The tail, or flagellum, consists of a 9 + 2 arrangement of microtubules and is surrounded by a plasma membrane (Fig. 5*D*).

Discussion

Most light microscopic studies on *Haliotis* have not categorised the various stages of spermatogenesis, apart from suggesting that there are four broad classes: spermatogonia,

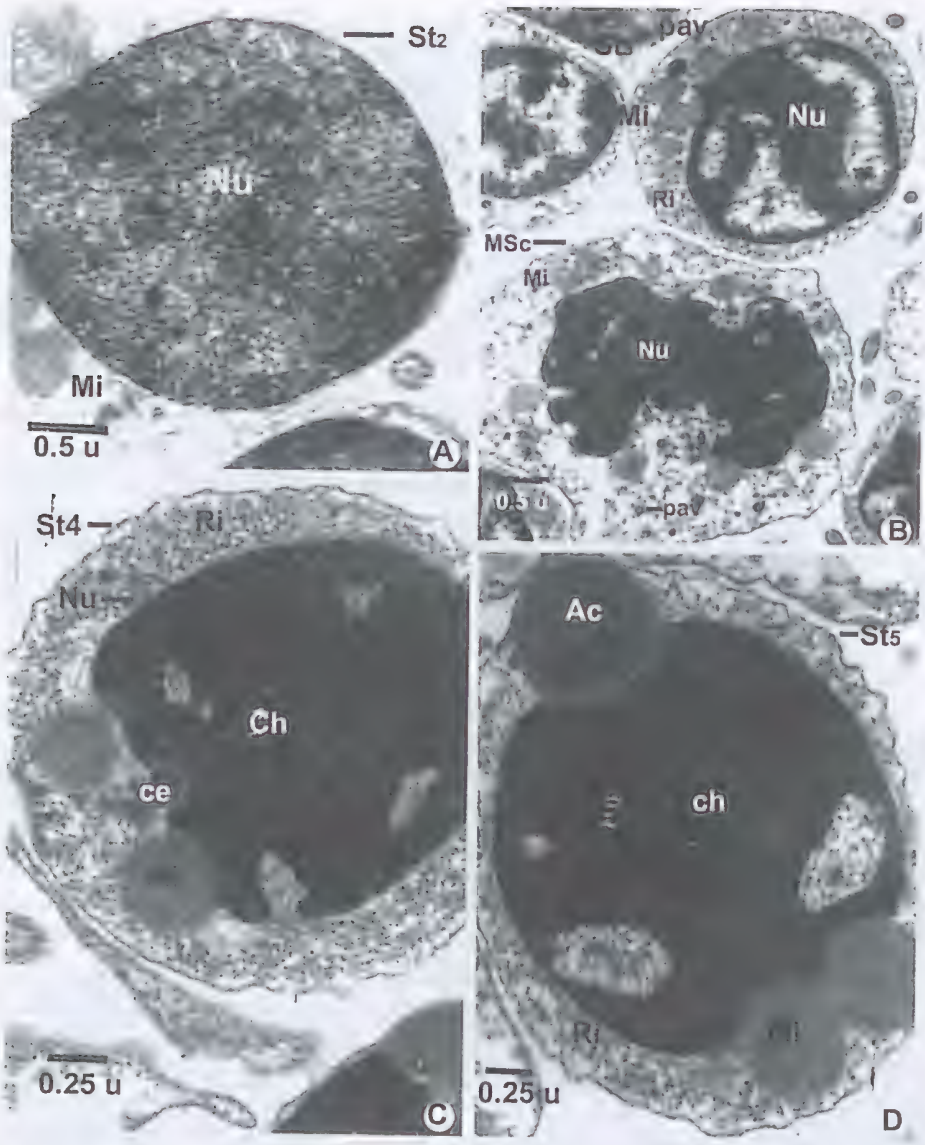

Fig. 4. *A*, Spermatid II (St_2) shows more condensed chromatin (ch) in a round nucleus (Nu). The cytoplasm exhibits fewer organelles. *B*, Spermatid III (St_3) contains a round nucleus. Chromatin is condensed into dark blocks. Mitochondria (Mi) begin to form a cluster. *C*, Spermatid IV (St_4) appears round in shape. The nucleus contains almost completely condensed chromatin. Mitochondria are located at the posterior border of the nucleus. Note the presence of centrioles (ce). *D*, Spermatid V (St_5) contains a nucleus with almost completely condensed chromatin. Note the fusion of proacrosomal vesicles (pav) into an acrosome (Ac). Mitochondria are larger and occupy the posterior border of the nucleus. Ri, Ribosome.

spermatocytes, spermatids and spermatozoa (Tomita 1967; Takashima *et al.* 1978). Apisawetakan *et al.* (1997) studied the gametogenic processes in *H. asinina* using the light microscope and classified spermatogenesis into 13 stages: spermatogonium, five stages of primary spermatocyte, secondary spermatocyte, four stages of spermatid and spermatozoa

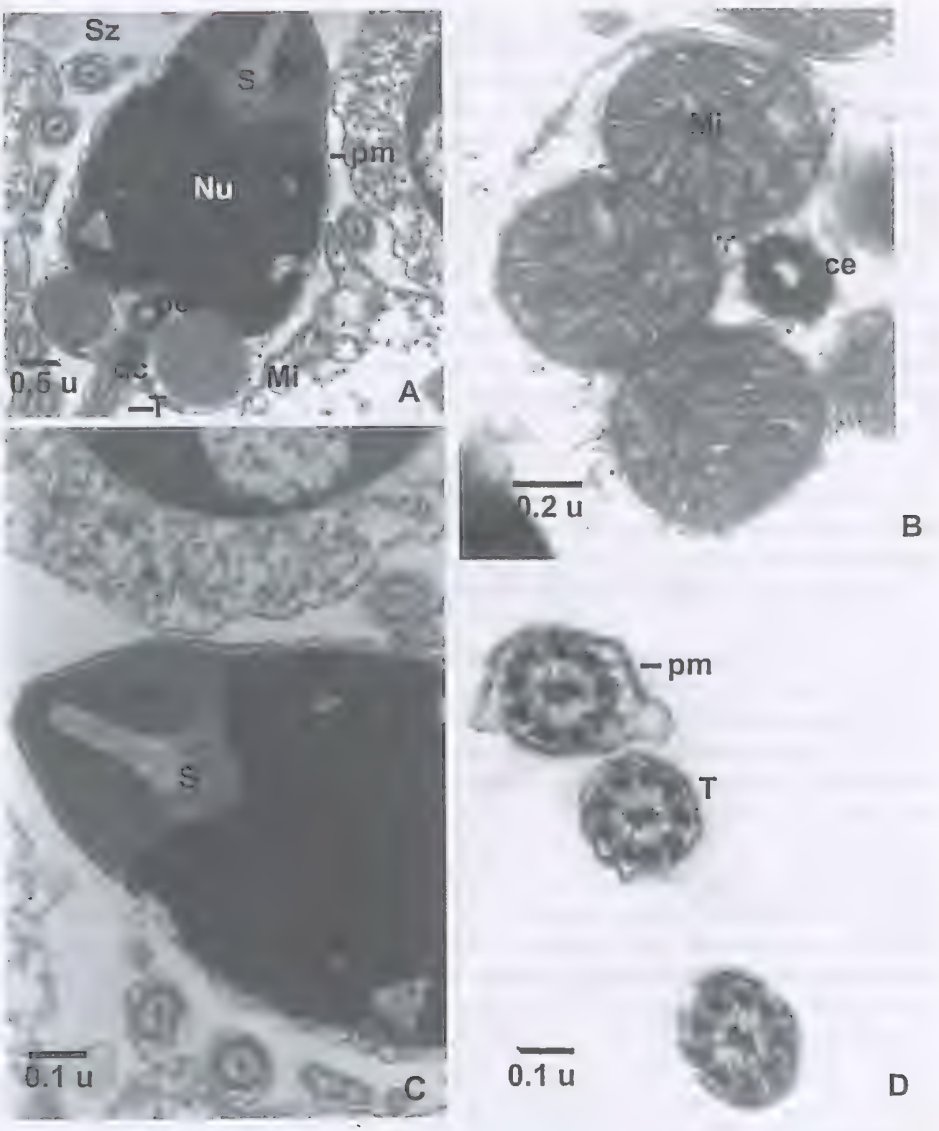

Fig. 5. Spermatozoa (Sz). *A*, Mature spermatozoa showing a fully elongated nucleus (Nu), acrosome (Ac) with subacrosomal space (S), proximal and distal centrioles (pc and dc, respectively) and a ring of mitochondria (Mi). *B*, High-power magnification of a ring of five mitochondria. *C*, High-power magnification of an acrosome. *D*, The tail (T) or flagellum consists of 9 + 2 arrangement of microtubules surrounded by a plasma membrane (pm). ce, Centriole.

(immature and mature). Sobhon *et al.* (2001) reported 14 stages of spermatogenesis based on their ultrastructural study: spermatogonium, six stages of primary spermatocyte, secondary spermatocyte, four stages of spermatid and spermatozoa (immature and mature). The present study determined 13 stages of spermatogenesis in *H. ovina*: the spermatogonium, five stages of the primary spermatocyte, the secondary spermatocyte, five stages of the spermatid and the spermatozoa. We could not observe the diakinetic stage of the primary spermatocyte, only the metaphase spermatocyte.

Like *H. asinina* (Sobhon *et al.* 1999, 2001), the spermatogonium of *H. ovina* is the earliest cell with a large nucleus containing a relatively large amount of euchromatin and a prominent nucleolus and little cytoplasm with free ribosomes and mitochondria. Spermatogonia divide mitotically to give rise to primary spermatocytes that pass through four stages of the first meiotic division. These prophase cells exhibit different forms of chromatin condensation. One remarkable characteristic of LSc, ZSc and PSc of both *H. ovina* and *H. asinina* is the presence of multiple proacrosomal vesicles in the cytoplasm. These vesicles begin to be synthesised in LSc and increase in PSc (Sobhon *et al.* 2001). In the MSc, the vesicles still appear quite numerous, whereas the chromatin becomes highly condensed into very large chromosomes. Thus, in this primitive gastropod, the proacrosomal vesicles are synthesised early in the LSc stage, even though they may be fused to form acrosomes much later in the late spermatid stages (Young and DeMartini 1970; Hodgson and Bernard 1986; Healy *et al.* 1998; Apisawetakan *et al.* 2000; Panasophonkul 2000; Sobhon *et al.* 2001). The fusion of multiple proacrosomal vesicles has been reported in several groups of vetigastropods, such as the trochids (Azevedo *et al.* 1985; Hodgson *et al.* 1990), bivalves (Hodgson and Bernard 1986) and scaphopods (Hou and Maxwell 1991). Although the very earliest stage of proacrosomal vesicle production was not observed in the present study, the ultimate source of these vesicles is undoubtedly the Golgi complex, as demonstrated previously for other vetigastropods (Azevedo *et al.* 1985; Healy and Harasewych 1992) and bivalves (Hodgson and Bernard 1986). Secondary spermatocytes, similar to those of *H. asinina* (Sobhon *et al.* 2001), have heterochromatin that exhibits a checkerboard pattern.

As mentioned earlier, there are five stages of spermatids in *H. ovina*, whereas Sobhon *et al.* (2001) described only four stages of spermatids in *H. asinina*. These classifications are based on size, chromatin granulation and condensation. Successive stages vary in size from 6 μm in St_1 to 2 μm in St_5. The major differences in the ultrastructure of developing spermatids of *H. ovina* and *H. asinina* are: (*1*) the formation of the acrosome begins in St_2 and is completed in St_3 for *H. asinina* (Sobhon *et al.* 2001), whereas this occurs at a later stage (St_5) in *H. ovina*; (*2*) all stages of spermatids in *H. ovina* retain a round shape, whereas they vary from round and oval (St_{1-3}) to ellipsoid (St_4) in *H. asinina* (Sobhon *et al.* 2001); (*3*) the acrosome of *H. ovina* appears slightly indented at the anterior region of the nucleus, whereas in *H. asinina* the acrosome only touches the nuclear envelope at the anterior end of the nucleus (Sobhon *et al.* 2001); and (*4*) the St_4 of *H. asinina* appears to be in a more advanced stage than that of *H. ovina* (i.e. there is a concentration of mitochondria in the posterior border of the nucleus and the centriole starts to form the axonemal complex of the tail (Sobhon *et al.* 2001); in *H. ovina*, only a concentration of mitochondria was found). There are two stages of spermatozoa (Sz_{1-2}) in *H. asinina* (Apisawetakan *et al.* 2000; Sobhon *et al.* 2001), whereas there is only one stage of spermatozoa in *H. ovina*. The Sz_1 is an immature spermatozoon with an acrosome and a short, developing tail, whereas Sz_2 is a mature spermatozoon.

The results of the present study show that the spermatozoa of *H. ovina* are very similar to those of *H. asinina*, except for the morphology of the sperm head. *Haliotis ovina* spermatozoa have a round to barrel-shaped head, whereas those of *H. asinina* are ellipsoid (Apisawetakan *et al.* 2000). In general, examination, to date, of spermatozoa in haliotid species shows that there are three types of sperm head: (*1*) short and globular or barrel-shaped (*H. ovina*; the present study); (*2*) ellipsoid (*H. laevigata* (Healy *et al.* 1998), *H. diversicolor supertexta* (Gwo *et al.* 1997) and *H. aquatilis* (Shiroya and Sakai 1992)); and (*3*) long and bullet-shaped (*H. discus* (Sakai *et al.* 1982; Usui, 1987), *H. rufescens*

(Lewis *et al.* 1980) and *H. midae* (Hodgson and Foster 1992)). Consequently, the sperm nuclei can also be classified into three types: round, ellipsoid and elongated. The chromatin of *H. ovina*, similar to that of *H. asinina* (Apisawetakan *et al.* 2000; Sobhon *et al.* 2001), appears to be of a granular type. This granular pattern of chromatin condensation can also be observed in other primitive gastropods, such as trochids (Hodgson *et al.* 1990; Healy 1996), scaphopods (Dufresne-Dube *et al.* 1993) and bivalves (Bozzo *et al.* 1993; Casas and Subirana 1994; Johnson *et al.* 1996). In *H. ovina* sperm, the acrosome is situated at the apex of the nucleus, as in *H. asinina* and also in many bivalves (Kubo 1977; Kubo and Ishikawa 1978; Apisawetakan *et al.* 2000; Sobhon *et al.* 2001).

The acrosome of *H. ovina* has an inverted cup shape, similar to those of *H. asinina*, *H. laevigata*, *H. diversicolor supertexta* and *H. aquatilis* (Gwo *et al.* 1997; Healy *et al.* 1998; Apisawetakan *et al.* 2000; Shiroya and Sakai 1992). In contrast, the acrosomes of *H. discus*, *H. rufescens* and *H. midae* are much longer and narrower (Lewis *et al.* 1980; Sakai *et al.* 1982; Hodgson and Foster 1992). The acrosomal core consists of a crystalline-like axis embedded within a moderately dense matrix that occupies the whole subacrosomal space. The core is much shorter. The crystalline material probably consists of actin filaments and associated proteins, as reported in other molluscs (Baccetti and Afzelius 1976; Shiroya *et al.* 1986; Tilney *et al.* 1987; Healy 1989). This acrosomal core may participate in the extension of the acrosomal process during the acrosomal reaction and fertilisation (Apisawetakan *et al.* 2000).

The tail of a *H. ovina* sperm consists of five globular mitochondria located at the posterior end of the nucleus surrounding a pair of centrioles. A long axoneme stretches backwards from the distal centriole, which is surrounded by mitochondria. The axoneme core consists of 9 + 2 doublets of microtubules surrounded by a plasma membrane. This type of tail and midpiece was also observed in *H. asinina* (Apisawetakan *et al.* 2000; Sobhon *et al.* 2001), *H. laevigata* (Healy *et al.* 1998), *H. aquatilis* (Shiroya and Sakai 1992), *H. diversicolor supertexta* (Gwo *et al.* 1997) and also in several other vetigastropods (Azevedo *et al.* 1985; Healy 1989; Hodgson *et al.* 1990; Healy and Harasewych 1992) and in many bivalves (Hodgson and Bernard 1990; Bozzo *et al.* 1993; Casas and Subirana 1994; Healy 1996; Johnson *et al.* 1996), all of which reproduce by external fertilisation. It is apparent that the sperm of those molluscs with external fertilisation do not require larger quantities of energy than those of molluscs with internal fertilisation. Such sperm usually have midpieces that contain large cylindrical or helical mitochondria (Jaramillo *et al.* 1986; Gallardo and Garrido 1989; Amor and Durfort 1990; Sretarugsa *et al.* 1991; Caceres *et al.* 1994).

Gwo *et al.* (1997) suggested that large species of Haliotidae usually possessed long sperm heads and small species contain short sperm heads. If this is the case, then *H. ovina* sperm should be classified into the latter group. Sperm of *H. ovina* bear certain similarities to those of *H. asinina*, except for the shape of the sperm head. Further studies need to be performed on the ultrastructure of haliotid sperm. We anticipate that continued comparative work in this field will help to shed further light not only on species relationships within the Haliotidae, but also on the validity of the many proposed subgenera.

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Gastropod phylogeny based on six segments from four genes representing coding or non-coding and mitochondrial or nuclear DNA

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Abstract

Significant differences remain between gastropod phylogenetic hypotheses based on morphological and molecular datasets. We collected additional data from three gene segments (*28S* rDNA expansion region *D1* (36 taxa plus two from GenBank), cytochrome *c* oxidase subunit 1 (35 species plus one from Genbank) and small nuclear RNA *U2* (24 species)). These were combined with data available for the same species for histone *H3* and two other segments of *28S* rDNA. Analyses of these data using cladistic, maximum likelihood or Bayesian methodologies were conducted in an attempt to resolve some of the differences between current hypotheses of gastropod relationships based on morphological and molecular data. The results were of particular interest in four areas. (*1*) Patellogastropoda in most analyses are included in a derived clade with some Vetigastropoda. In an analysis with *Nautilus* as the sole outgroup, transversions weighted threefold as costly as transitions and, with third codon position data ignored, Patellogastropoda are excluded from an otherwise monophyletic Gastropoda. (*2*) Cocculiniformia was never monophyletic in our analyses, although this possibility is not statistically rejected. (*3*) *Nerita*, the only representative of Neritopsina in this dataset, is placed anomalously in most analyses, but is, in a few cases, shown as a sister-group to the Apogastropoda, in accord with some morphological hypotheses. (*4*) Heterobranchia is rarely monophyletic in our analyses owing to the variable placement of the architectonicoid *Philippea*. This genus, even judged by the high levels of divergence within Heterobranchia, has undergone extreme rates of substitution. The Euthyneura is invariably monophyletic and nearly always included in a clade with the valvatoidean *Cornirostra* as its sister-group.

Additional keywords: DNA sequence, heterobranch, multiple gene, Neritopsina, patellogastropod.

Introduction

Recent phylogenetic investigations of gastropods have used a variety of different datasets, ranging from shell morphology (including fossils, protoconch morphology and shell structure; Bandel 1997; Frýda, 1999; Wagner 2002; for a review, see Wagner 2001), ultrastructure (Ponder and Lindberg 1997; Künz and Haszprunar 2001), development (Freeman and Lundelius 1992; van den Bigelaar 1996; van den Bigelaar and Haszprunar 1996; Lindberg and Guralnick 2001), pallial cavity structures (Haszprunar 1988*a*; Ponder and Lindberg 1996, 1997; Lindberg and Ponder 2001), overall morphology (Haszprunar 1988*a*; Ponder and Lindberg 1996, 1997; Barker 2001), mitochondrial gene order (Kurabayashi and Ueshima 2000*a*, 2000*b*) and molecular sequences.

These studies show general agreement for the recognition of five major groups within Recent gastropods: (*1*) the Patellogastropoda (or Docoglossa), being the true limpets; (*2*) the Vetigastropoda (trochids, haliotids, fissurellids etc.); (*3*) the Neritopsina (or Neritimorpha), the nerites and relatives; (*4*) the Caenogastropoda (most of the 'mesogastropods', including the architaenioglossan taxa, and the neogastropods); and (*5*) the Heterobranchia (or Heterostropha as used by some palaeontologists (Bandel 1997),

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whereas others (Ponder and Warén 1988) use Heterostropha as a paraphyletic subgroup within the Heterobranchia). The Caenogastropoda and Heterobranchia form the Apogastropoda in Ponder and Lindberg's (1997) sense. This taxon was originally introduced by Salvini-Plawen and Haszprunar (1987) to include the caenogastropods and only the basal heterobranchs, or 'allogastropods', in their usage, making it paraphyletic. An additional extant group, the Cocculiniformia (for a review, see Haszprunar 1998), is sometimes recognised and placed near the base of the gastropods.

The first three groups and the Cocculiniformia comprise the paraphyletic 'archaeogastropods', whereas the first four groups comprise the paraphyletic 'prosobranchs'. The Heterobranchia, as first recognised by Haszprunar (1985*a*), contains the euthyneurans, a grouping of two taxa previously given subclass rank (the opisthobranchs and the pulmonates), as well as a number of assumed more 'primitive' members, all of which were included within the 'prosobranchs' at some time. These latter groups comprise the paraphyletic 'Heterostropha' (*sensu* Ponder and Warén 1988) or 'Allogastropoda' (Haszprunar 1985*b*; Bandel 1997).

Hickman (1988) advocated restricting the usage of the name Archaeogastropoda to the Vetigastropoda, whereas some (Bandel 1982, 1997; Bandel and Geldmacher 1996) formally use the same name for a group encompassing the patellogastropods together with the vetigastropods and the Cocculiniformia (i.e. excluding the Neritopsina), based primarily on their possession of a similar larval shell. However, the artificial nature of such a grouping is recognised (Frýda 1999). In stark contrast with this view is the idea that the patellogastropods represent the sister-taxon to the rest of the gastropods, a result suggested by most recent morphological studies (Golikov and Starobogatov 1975; Graham 1985; Haszprunar 1988*a*; Ponder and Lindberg 1996, 1997; Sasaki 1998; Barker 2001). It was on this basis that Ponder and Lindberg (1997) introduced the Eogastropoda, to encompass the Patellogastropods and their assumed coiled ancestors, and Orthogastropoda for the remainder of the gastropods.

Gastropods date from the early Cambrian, although there is considerable speculation over which of the small univalve fossils in that period represent torted gastropods or monoplacophorans. For example, Parkhaev (2001) assigns Helcionelloidea *s.s.* to Gastropoda, whereas members of this group are often considered to be monoplacophorans (Wen 1990; Peel 1991).

Only recently have individual molecular investigations of gastropod phylogeny included examples from nearly all critical taxa in the class (McArthur and Koop 1999; Colgan *et al.* 2000). The genes now available for a broadly representative set of taxa include histone *H3* (Colgan *et al.* 2000) and four regions of the *28S* rDNA gene. These are the *D1* (Tillier *et al.* 1992, 1994; McArthur and Koop 1999), *D6* (Rosenberg *et al.* 1997; McArthur and Koop 1999) and *D4–5* ('28SA' in Colgan *et al.* 2000) expansion regions and a section near the *D7* area including a new expansion region ('28SB' in Colgan *et al.* 2000). With some notable exceptions, major groups are supported or weakly contradicted in molecular investigations. Nevertheless, the most comprehensive analysis (Colgan *et al.* 2000) is based on relatively short sequences (less than 900 aligned base positions), so it is not surprising that there are still disagreements between molecular and morphological (Haszprunar 1988*a*; Ponder and Lindberg 1997) assessments of gastropod relationships (Fig. 1). The collection of more molecular and morphological data offers the best chance of resolving such disagreements, although data from fossils are also being examined rigorously and may well assist in further elucidating gastropod phylogeny (Wagner 2001).

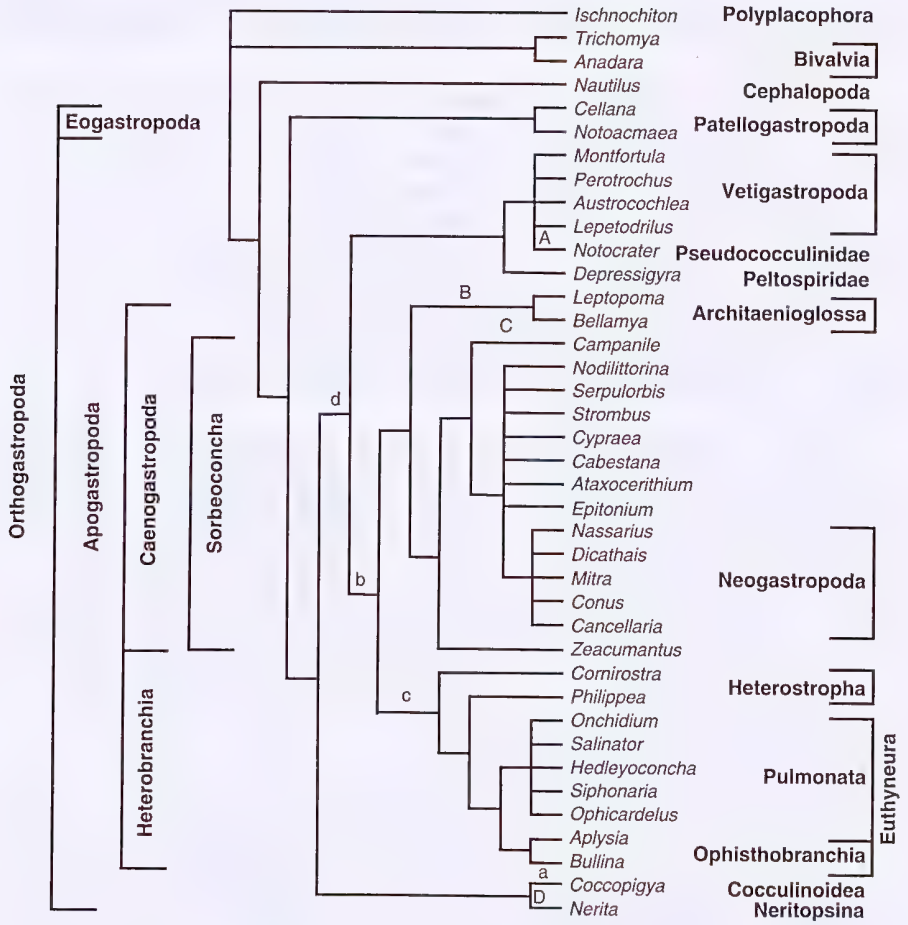

Fig. 1. Gastropod phylogeny based on the morphological analyses of Ponder and Lindberg (1997). Families not included in their analyses are placed according to their general taxonomic classification (i.e. Cancellariidae and Mitridae in Neogastropoda, Onchidiidae, Siphonariidae, Ellobiidae and Charopidae in the Pulmonata). Taxa studied by Ponder and Lindberg (1997) but not here are pruned from the tree. Names of higher categories follow Ponder and Lindberg (1997). Differences of this topology from the Haszprunar (1988) topology are indicated. An upper case letter on a clade in the Ponder and Lindberg (1997) tree indicates that it is shown at the point specified by the corresponding lower case letter in the Haszprunar tree.

In the present paper, we report analyses of an enlarged molecular dataset principally addressed to four areas of disagreement with morphologically based hypotheses, namely: (*1*) the position of the Patellogastropoda; (*2*) the monophyly and relationships of Cocculiniformia; (*3*) the relationships of Neritopsina; and (*4*) the monophyly of the Heterobranchia. Parts of two genes not previously used in overall gastropod phylogeny (cytochrome *c* oxidase subunit 1 (*CO1*) and small nuclear *U2* RNA (*snU2* RNA)) have been sequenced and new sequences from the *D1 28S* rDNA expansion region have been collected for most species studied in Colgan *et al.* (2000). The new genes extend the range of gene types used in gastropod phylogeny because they are respectively mitochondrial coding (*CO1*) and nuclear non-coding (*snU2* RNA). The *snU2* RNA is a component of the

spliceosome (Guthrie and Patterson 1988) that has previously provided useful characters for higher-level phylogenetic studies of Arthropoda (Colgan *et al.* 1998) and Polychaeta (Brown *et al.* 1999).

The division of Gastropoda into Eogastropoda and Orthogastropoda has not been supported in comprehensive molecular studies to date. In Colgan *et al.* (2000), Patellogastropoda plus a cocculiniform limpet (Cocculinoidea: *Coccopigya*) was a sister-group to the remainder of the studied species. A similar topology is seen in analyses of the approximately 450 (aligned) base pairs of *18S* rDNA in the compiled dataset of Harasewych and McArthur (2000), where Patellogastropoda plus a monophyletic Cocculiniformia is a sister-group to all other gastropods. In McArthur and Koop (1999), where Cocculiformia are not represented, Patellogastropoda is a sister-group to Apogastropoda. Statistically, no placement of Patellogastropoda as a sister-group to another major clade has a significantly higher likelihood than any other in the McArthur and Koop (1999) analyses.

Monophyly of 'cocculiniform' limpets is one of the major areas of disagreement between the Haszprunar (1988*b*) and Ponder and Lindberg (1996, 1997) morphological topologies. The two main constituent groups, Cocculinoidea and Lepetelloidea, comprise a monophyletic Cocculiniformia (Haszprunar 1988*a*; 1998) or are diphyletic (Ponder and Lindberg 1996, 1997). The molecular datasets also disagree. Cocculiniformia are diphyletic in Colgan *et al.*(2000), *Notocrater houbricki* (Lepetelloidea) being placed in Vetigastropoda and *Coccopigya hispida* (Cocculinoidea) with Neritopsina in accordance with Ponder and Lindberg (1997). Cocculiniformia represented by *Cocculina messingi* (Cocculinoidea) and *N. houbricki* are monophyletic (albeit with weak support) in Harasewych and McArthur (2000), using partial *18S* rDNA data, and are a sister-group to Patellogastropoda.

The relationship of the Neritopsina (or Neritimorpha) to the rest of the gastropods is unresolved with two main scenarios: they are either a sister-group to the vetigastropods and the apogastropods or a sister-group to the apogastropods. Frýda (1999), Bandel and Frýda (1999) and Bandel (2000) have discussed the fossil evidence for the evolution of this group since its first undoubted appearance in the Late Silurian–Devonian (428–374 million years ago; Frýda and Blodgett 2001), although earlier origins have been argued.

Within the Heterobranchia, Euthyneura, comprising two of the three classes (Opisthobranchia and Pulmonata) in Thiele's (1925, 1929–31) classification, are monophyletic in most recent molecular analyses where few taxa are used, but not in studies with larger taxonomic samples (Thollesson 1999; Dayrat *et al.* 2001). In morphological analyses involving Recent taxa, Heterobranchia is the sister-clade to Caenogastropoda (Haszprunar 1988*a*; Ponder and Lindberg 1996, 1997). In McArthur and Koop (1999), the only included heterostrophan (*Valvata* sp.) is a sister-taxon to Euthyneura. In Colgan *et al.* (2000), two representatives of the group are included. They are monophyletic but not a sister-group to Euthyneura, possibly owing to the high degree of autapomorphy in their sequences, this being particularly notable for the architectonicoid species *Philippea lutea* (see below).

Materials and methods

Materials and molecular methods

The taxa used, collection and registration data of specimens, methods and tissue types used for DNA extraction are given in Colgan *et al.* (2000). Our naming of higher groups follows Ponder and Lindberg (1997). Specimen voucher numbers are given in Table 1.

Primer pairs for *U2* are given in Colgan *et al.* (1998). The primers for *CO1* were as follows: Cox AF, CWAATCAYAAAGATATTGGAAC (41); Cox AR, AATATAWACTTCWGGGTGACC (725); and Cox 623R, GGTAARTYTATTGTAATAGCWCC (623). The figures in parentheses refer to the 3′ end of the oligonucleotide in the sequence of *Lumbricus terrestris* (Boore and Brown 1995; GenBank accession LTU24570). Primers Cox AF and AR were used together to produce a 690 bp product. Where a product was not obtained using this pair, Cox 623R was used with Cox AF to give a 626 bp product.

The primers for the *D1* expansion region were as follows: 28S D1F, ACCCSCTGAAYTTAAGCAT (43); 28S D1R, AACTCTCTCMTTCARAGTTC (406). Figures in parentheses refer to the 3 end of the oligonucleotide in the mouse *28S* rDNA sequence (X00525; Hassouna *et al.* 1984). Primer D1F was taken from Macarthur and Koop (1999) and D1R was designed from an alignment of *Ascaris lumbricoides* (U94751), *Drosophila melanogaster* (M21017, M29800) and *Mus musculus* (X00525) sequences.

The basic polymerase chain reaction (PCR) profile was as follows: 95°C for 5 min, 43–54°C for 45 s, 72°C for 1 min for one cycle; 95°C for 30 s, 43–54°C for 45 s, 72°C for 1 min for 30–34 cycles; and 95°C for 30 s, 45–60°C for 45 s, 72°C for 5 min for the final cycle. The annealing temperatures varied according to the primers' specificity for the different taxa and were usually 50–52°C for *U2*, 52–54°C for *D1 28S* rDNA and 43–45°C for *CO1*. To obtain PCR products from difficult samples, 20 µL GeneReleaser™ (Bioventures, Murfreesboro, TN, USA) was added to the DNA template and microwaved for 6 min. The remaining PCR mix was immediately added and cycling commenced. Reaction products were resolved on 2% agarose gels containing ethidium bromide. All single band products were purified using the QIAquick™ PCR Purification Kit (Qiagen, Venlo, The Netherlands) and, where multiple band products were obtained, the correct sized band was excised from 2% low-melt agarose in TAE buffer (0.04 M Tris , 0.001 M EDTA (pH 8.0), 0.02 M glacial acetic acid) and purified using the same kit. Products were sequenced in both directions by the Applied Biosystems (ABI®; Norwalk, CT, USA) 310 DNA Sequencing System using the DyeDeoxy™ Terminator sequencing method (Big Dye™ version 1 or 2; ABI), according to the manufacturer's instructions. The consensus sequence for each individual was obtained by reconciling forward and reverse sequences using Sequence Navigator (ABI).

The GenBank accession numbers of sequences used in Colgan *et al.* (2000) are AF033716–AF033794 for *28SA* rRNA and *28SB* rRNA and AF033675–AF033715 for *H3*. *Nautilus pompilius CO1* data are from AF000054 (Carlini and Graves 1999). Sequences for Viviparidae (U75863) and Cornirostridae (U75862) were obtained from GenBank (McArthur and Koop 1999). The GenBank accession numbers for the new sequences are AY296815–AY296850 for *CO1*, AY296873–AY296909 for *28S D1* and AY296851–AY296872 for *snU2* RNA.

Phylogenetic analysis

Sequences were aligned using the default values in CLUSTAL W (Thompson *et al.* 1994). The CLUSTAL output was inspected and, where required, indels were edited by hand. The *CO1* and *U2* sequences required no modification, but some regions of the *D1* segment were altered. These were specified as regions of uncertain alignment and were not used for analyses. All bases in the *28SA* and *H3* alignments from Colgan *et al.* (2000) were used. The region of uncertain alignment in *28S* rDNA B in Colgan *et al.* (2000) was not used. The alignments are available from the authors and as Accessory Material from the journal's website.

PAUP* 4.0b10 (Swofford 2000) was used for maximum parsimony analysis by conducting heuristic searches (100 iterations with random input order) with the default options (unless otherwise stated below). All characters were assumed unordered and indels treated as unknown in all reported analyses. One hundred bootstrap pseudoreplicates (Felsenstein 1985) were conducted with 20 random input replicates at each replicate to estimate support for nodes. Bremer decay indices were calculated using AutoDecay version 3.03 (Eriksson and Wikström 1996)

Maximum likelihood analyses of a reduced taxon set were performed using 10 random addition sequences for heuristic searches with the following settings. The number of substitution types was two, with the transition/transversion ratio estimated by maximum likelihood. Empirical nucleotide frequencies were assumed. The assumed proportion of invariable sites was zero, with a gamma discrete approximation (with shape parameter 0.5) using four rate categories. Another ML analysis was conducted with the same assumptions except that the maximum parsimony trees were used to start the analysis, branch swapping was by subtree pruning and reconnection, and five replicates were used. Quartet puzzling (100 000 steps) was performed using PAUP with the same likelihood settings, entering the transition/transversion ratio estimated during the likelihood searches by hand.

A Bayesian analysis was conducted with the program MrBayes (Huelsenbeck and Ronquist 2001) using the same likelihood parameters as the maximum likelihood analysis and sampling a tree every 100 steps

Table 1. Classification, species and voucher details

Higher-order grouping	Species	Specimen no.	Missing data
POLYPLACOPHORA Neoloricata			
Ischnochitonina, Ischnochitonidae	*Ischnochiton* (*Ischnoradsia*) *australis* (Sowerby, 1840)	C.203201	
BIVALVIA Pteriomorpha			
Arcoidea, Arcidae	*Anadara trapezia* (Deshayes, 1839)	C.203202	
Mytiloidea, Mytilidae	*Trichomya hirsuta* (Lamarck, 1819)	C.203203	
CEPHALOPODA Nautiloida			
Nautilida, Nautiloidea	*Nautilus scrobiculatus* Lightfoot, 1786	USNM 885678	
GASTROPODA			
EOGASTROPODA Patellogastropoda			
Patellina, Patelloidea, Patellidae	*Cellana tramoserica* (Holten, 1802)	C.203204	
Nacellina, Acmaeoidae, Lottiidae	*Notoacmaea petterdi* (T. Woods, 1876)	C.203205	*CO1*
ORTHOGASTROPODA			
Neomphaloidea, Peltospiridae	*Depressigyra globulus* Warén & Bouchet, 1989	T. HYS-202	
Cocculiniformia			
Cocculinoidea, Cocculinidae	*Coccopigya hispida* Marshall, 1986	NMNZ M.75188	
Pseudococculinoidea, Pseudococculinidae	*Notocrater houbricki* McLean & Harasewych, 1995	USNM 888656	*U2*
Vetigastropoda			
Fissurelloidea, Fissurellidae	*Montfortula rugosa* (Quoy & Gaimard, 1834)	C.203206	
Lepetodriloidea, Lepetodrilidae	*Lepetodrilus fucensis* McLean, 1988	T. F20-A2413	*U2*, *CO1*
Pleurotomaroidea, Pleurotomariidae	*Perotrochus midas* Bayer, 1965	USNM 888645	
Trochoidea, Trochidae	*Austrocochlea porcata* (A. Adams, 1851)	C.203207	
Neritopsina			
Neritoidea, Neritidae	*Nerita atramentosa* Reeve, 1855	C.203208	*U2*
Caenogastropoda			
'Architaenioglossa'			
Cyclophoroidea, Cyclophoridae	*Leptopoma perlucida* (Grateloup, 1840)	QM MO31142	
Ampullarioidea, Viviparidae	*Bellamya heudi guangdungensis* (Kobelt, 1906)	C.203209	*U2*
Sorbeoconcha 'Neotaenioglossa'			
Cerithioidea, Batillariidae	*Zeacumantus subcarinatus* (Sowerby, 1855)	C.203210	
Campaniloidea, Campanilidae	*Campanile symbolicum* Iredale, 1917	C.203211	

Hypsogastropoda			
Littorinoidea, Littorinidae	*Nodilittorina unifasciata* (Gray, 1826)	C.203212	
Vermetoidea, Vermetidae	*Serpulorbis* sp.	C.203213	*U2*
Stromboidea, Strombidae	*Strombus* (*Conomurexi*) *luhuanus* Linnaeus, 1758	C.203214	*U2*
Cypraeidae, Cypraeoidea	*Cypraea* (*Monetaria*) *annulus* Linneaus, 1758	C.203215	*U2*
Tonnoidea, Ranellidae	*Cabestana spengleri* Perry, 1811	C.203216	*U2*
Sorbeoconcha *'Ptenoglossa'*			
Triphoroidea, Cerithiopsidae	*Ataxocerithium* sp.	C.203217	*U2*
Janthinoidea, Epitoniidae	*Epitonium* cf. *jukesianum* (Forbes, 1852)	C.203218	
Sorbeoconcha Neogastropoda			
Buccinidae	*Nassarius* (*Plicarcularia*) *burchardi* (Dunker *in* Philippi, 1849)	C.203219	*U2*
Muricidae	*Dicathais orbita* (Gmelin, 1791)	C.203220	
Mitridae	*Mitra cucumerina* Lamarck, 1811	C.203221	*U2*
Cancellarioidea, Cancellariidae	*Cancellaria* (*Sydaphera*) *undulata* Sowerby, 1849	C.203222	*U2*
Conoidea, Conidae	*Conus miles* Linnaeus, 1758	C.203223	
Heterobranchia 'Heterostropha'			
Valvatoidea, Cornirostridae	*Cornirostra pellucida* (Laseron, 1954)	C.203224	
Architectonicoidea, Architectonicidae	*Philippea lutea* (Lamarck, 1822)	C.203225	
Heterobranchia Euthyneura			
Ophisthobranchia			
Cephalaspidea, Hydatinidae	*Bullina lineata* (Gray, 1825)	C.203226	*U2*
Notaspidea, Aplysiidae	*Aplysia* cf. *juliana* Quoy & Gaimard, 1832	C.203227	
Pulmonata			
Onchidiidae	*Onchidium* sp.	C.203228	
Amphiboloidea, Amphibolidae	*Salinator solida* (Schacko, 1878)	C.203229	*U2*
Siphonarioidea, Siphonariidae	*Siphonaria zelandica* (Quoy & Gaimard, 1833)	C.203230	
Ellobioidea, Ellobiidae	*Ophicardelus ornatus* (Férussac, 1821)	C.203231	
Stylommatophora, Charopidae	*Hedleyoconcha delta* (Pfeiffer, 1857)	QM MO6068	

Unless otherwise specified, samples are identified by their registration in the malacological collection of the Australian Museum. Other abbreviations are as follows: NMNZ, National Museum of New Zealand, Wellington; QM, Queensland Museum, Brisbane; USNM, National Museum of Natural History, Washington, DC; T, Tunnicliffe Collection).

An entry in the 'Missing data' column indicates that the specified gene was not scored for that taxon.

Sequences from GenBank are specified by accession number and referenced in the text.

along a 100 000 step Markov chain. Only two simultaneous chains were run, owing to computer memory restrictions, and the first 43 300 steps were discarded because convergence to an area of stable likelihood did not occur until this time.

All analyses listed below, except (*ix*)–(*xi*), were conducted with maximum parsimony.

(*i*) All data, excluding areas of uncertain alignment in the *28S* rDNA.
(*ii*) All data, excluding areas of uncertain alignment in the *28S* rDNA and third codon positions.
(*iii*) All data, excluding areas of uncertain alignment in the *28S* rDNA, transversions weighted threefold as costly as transitions.
(*iv*) All data, excluding areas of uncertain alignment in the *28S* rDNA and third position data, transversions weighted threefold as costly as transitions.
(*v*) All data, excluding areas of uncertain alignment in the *28S* rDNA, transversions weighted threefold as costly as transitions; *Nautilus* was used as the only outgroup.
(*vi*) All data, excluding areas of uncertain alignment in the *28S* rDNA and third codon position data, transversions weighted threefold as costly as transitions; *Nautilus* was used as the only outgroup.
(*vii*) All data, excluding areas of uncertain alignment in the *28S* rDNA, and excluding *Philippea lutea*.
(*viii*) All data, excluding areas of uncertain alignment in the *28S* rDNA and third codon position data, and excluding *Philippea lutea*.
(*ix*) A maximum likelihood analysis of a reduced dataset using random taxon addition and excluding areas of uncertain alignment in the *28S* rDNA.
(*x*) A maximum likelihood analysis using maximum parsimony starting trees excluding areas of uncertain alignment in the *28S* rDNA.
(*xi*) A Bayesian analysis of all data excluding areas of uncertain alignment.

Although analyses of individual genes are not reported in detail, they were conducted to confirm that there was no cross-contamination between sequences within this study or the inclusion of sequences from other material treated in this laboratory.

MacCLADE (Maddison and Maddison 1992) was used to set character types and substitution weights and to compare trees with the 'winning-sites test' (Prager and Wilson 1988) using the 'compare two tree files' option. Files containing all trees from each analysis were used. One tree (or set of trees) was preferred to another if the number of characters for which it had less steps than the alternative tree(s) was significantly greater than the number of such characters for the alternative. Probabilities were estimated using a two-tailed normal approximation.

Results

The number of aligned bases excluding the areas of uncertain alignment, the number of variable characters and the number of parsimony informative characters (in order for the individual gene segments) were: *D1 28S* rDNA, 317, 154 and 113 respectively; *28SA*, 255, 111 and 71 respectively; *28SB*, 256, 73 and 47 respectively; *CO1*, 567, 400 and 318 respectively; histone *H3* 274, 121 and 107 respectively; and *snU2* RNA 131, 56 and 41 respectively.

The mean GC content of the studied species for *snU2* RNA was lower (46.11%) than the other non-coding RNAs and notable differences were observed between the three *28S* rDNA segments: 57.35% for the *D1* region, 50.67% for *28SA* and 48.67% for the *28SB* region. The GC content was 58.42% for histone *H3*. The percentage of GC in *CO1* was very low (38.36%), but increased to 45.37% with the exclusion of third-position bases, consistent with the usual pattern for animal mitochondrial coding DNA (Wirth *et al.* 1999). Chi-square tests suggest that the percentage nucleotide composition was homogeneous within the studied species for *H3* ($P = 0.571$), *U2* ($P \approx 1$) and all *28S* segments ($P \approx 1$ for *D1 28S* rDNA, *28SA* and *28SB*), but was significantly inhomogeneous for *CO1* ($P < 0.001$). This inhomogeneity was due to third codon position data; when these data were omitted, the hypothesis of compositional homogeneity was not rejected ($P \approx 0.999$).

The average transition to transversion ratios for the six gene segments based on the Kimura two-parameter distance, and ignoring the areas of uncertain alignment and pairwise

comparisons without transversions, were: 1.792 ± 1.560 for *U2* (range range 0–13.31); 1.260 ± 0.600 for *H3* (range 0.348–5.260); 1.276 ± 0.874 for *D1 28S* rDNA (range 0–12.036); 3.017 ± 2.790 for *28SA* (range 0–26.065); 2.555 ± 3.602 for *28SB* (range 0–18.622); and 1.063 ± 0.306 for *CO1* (range 0.456–2.345). Ratios were also examined omitting the third position of *H3* and *CO1*. For *H3*, the average was 1.980 ± 1.028 (range 0.279–9.793). For *CO1*, the average was 1.372 ± 1.376 (range 0.279–21.186).

Incongruence length difference analysis for the data set for analysis (*i*) with 100 replicates returned a probability of 0.27 that the phylogenetic implications of the various gene segments do not differ. For other analyses, the probabilities are: (*ii*) 0.99; (*iii*) 0.99; (*iv*) 0.01; (*v*) 0.99; (*vi*) 0.99; (*vii*) 0.01; and (*viii*) 0.62. Summaries of maximum parsimony analyses for individual genes are given in Table 2. Generally, few clades receive bootstrap support greater than 50% in these analyses, so the results are not discussed in detail.

Details of the various analyses, including the number of maximum parsimony trees, the consistency index, the length of the trees and the minimum length of trees satisfying the Ponder and Lindberg (1997) topology, are presented in Table 3. The maximum parsimony trees for analyses (*i*), (*ii*), (*iii*), (*vi*) and (*ix*) are presented in Figs 2–6. The results for other analyses are compared with these figures below. Their bootstrap supported clades are indicated in Table 2.

Figure 2 shows one of two maximum parsimony trees for analysis (*i*). The topology of the second differs only above point A on the figure (with bolded branches found in both trees). The topology with *Philippea* removed (analysis (*vii*)) is the same as for analysis (*i*) (omitting *Philippea*) at nodes below the arrows on Fig. 2. The topologies differ above the arrowheads, notably in that Opisthobranchia and Pulmonata are monophyletic sister-groups in analysis (*vii*). Bootstrap supported clades are the same as for analysis (*i*) and have similar percentages.

The topology for analysis (*viii*) is the same as for analysis (*ii*) (with the removal of *Philippea*) at nodes below the arrow on Fig. 3, except that Patellogastropoda is a sister-group to the clade comprising (*Montfortula*, *Austrocochlea*, *Notocrater* and *Lepetodrilus*). Branches above the arrow seen in the strict consensus for analysis (*ii*) and for analysis (*viii*) are indicated in bold.

Figure 4 shows one of the two maximum parsimony trees for analysis (*iii*). The second tree differed in the placement of some taxa in the area between *Nodilittorina* and *Bellamya*, with *Nerita* shown in the equivalent position to *Bellamya* in the first tree. In analysis (*v*) *Nautilus*, *Coccopigya* and the other gastropods form a basal trichotomy. The gastropods are then divided into: (a) Euthyneura; and (b) the remaining taxa. Group (b) is further divided: (b.1) *Leptopoma* and *Campanile*; and (b.2) other Caenogastropoda, Vetigastropoda, Patellogastropoda and *Philippea*.

The topology for analysis (*iv*) is similar to that for analysis (*vi*). Addition of the other outgroup taxa in analysis (*iv*) places the root of the tree at the position marked by A in Fig. 5.

The maximum likelihood topology of the reduced taxon set is shown in Fig. 6. The estimated transition/transversion ratio on which this is based was 1.64. The estimated ratio for analysis (*x*) was 1.62. Analysis (*x*) produced two trees differing only in some placements within Caenogastropoda. The primary dichotomy lay between a group comprising Patellogastropoda, *Notocrater* and the Vetigastropoda except *Perotrochus* and the other gastropods. Caenogastropoda was monophyletic except that it anomalously included *Nerita* and that *Leptopoma* was grouped with *Nautilus* plus *Philippea* as a sister-group to a clade comprising *Coccopigya*, *Pleurotomaria* and *Depressigyra*. Euthyneura was monophyletic

Table 2. Summary of results for individual segments

Analysis	Bootstrap-supported clades	No. trees	*CI*	Length
28S D1	(*Strombus*, *Conus*) 90%; Eut plus *Cornirostra* and *Coccopigya* 79%; Eut plus *Coccopigya* 55%; (*Onchidium*, *Ophicardelus*) 82%; (*Bullina*, *Coccopigya*) 56%	1001	0.497	531
28SA	Biv 70%; Pat 100%; Eut 53%; (*Serpulorbis*, *Epitonium*, *Mitra*) 58%; (*Onchidium*, *Salinator*) 55	600	0.645	262
28SB	Pat 100%; (*Nodilittorina*, *Serpulorbis*, *Epitonium*, *Ataxocerithium*) 90%	8	0.690	142
28S (combined)	Gastropoda plus *Nautilus* 52%; Pat 100%; (*Montfortula*, *Austrocochlea*, *Lepetodrilus*) 62%; (*Austrocochlea*, *Lepetodrilus*) 51%; (*Nodilittorina*, *Serpulorbis*, *Epitonium*, *Ataxocerithium*) 73%; (*Serpulorbis*, *Epitonium*) 82%; (*Strombus*, *Conus*) 64%; (*Onchidium*, *Ophicardelus*) 73%; (*Hedleyoconcha*, *Onchidium*, *Ophicardelus*) 53%	5	0.525	1027
H3	Pat 56%	2	0.236	905
H3 (no third position)	(*Leptopoma*, *Strombus*) 52%; (*Montfortula*, *Austrocochlea*, *Notocrater*, *Lepetodrilus*) 72	320	0.500	70
U2	Pat 68%; (*Montfortula*, *Austrocochlea*) 86%; (*Leptopoma*, *Campanile*) 53%; Het except *Hedleyoconcha* 51%; (*Salinator*, *Onchidium*) 51%	20	0.503	183
CO1	Biv plus *Philippea* 91%; (*Nautilus*, *Leptopoma*) 54%; (*Coccopigya*, *Depressigyra*) 60%; Eut 71%; Eut except *Siphonaria* 78%; Pul except *Siphonaria* 57%	3	0.253	3175
CO1 (no third position)	(*Coccopigya*, *Depressigyra*) 53%; (*Trichomya*, *Philippea*) 54%; Biv plus *Philippea* 91%; Eut plus Biv plus *Philippea* 69%; Eut 73%; Eut except *Siphonaria* 81%	16	0.411	852

The columns show the number of maximum parsimony trees, their consistency index and length. The grouping of taxa by 'plus' does not necessarily indicate reciprocal monophyly.
Biv, Bivalvia; Pat, Patellogastropoda; Het, Heterobranchia; Eut, Euthyneura; Pul, Pulmonata.

Table 3. Statistics for individual analyses

Analysis	Trees	*CI*	Length	Ponder/Lindberg length	Winning sites		Normal deviate
(*i*)	2	0.298	5533	5603	179	92	5.28***
(*ii*)	4	0.447	2273	2347	68	25	4.46***
(*iii*)	1	0.277	10559	10842	217	127	4.85***
(*iv*)	12	0.455	4132	4273	87	26	5.74***
(v)	1	0.285	9280	9443	177	152	1.38
(*vi*)	28	0.440	2307	2383	73	27	4.60***
(*vii*)	4	0.298	5275	5411	164	81	5.30***
(*viii*)	10	0.453	2096	2143	65	31	2.14*
Eogastropoda/Orthogastropoda	4	0.298	5565		95	74	1.61
Heterobranchia	6	0.297	5556		119	101	1.21
Cocculiniformia	1	0.297	5551		140	114	1.63

The winning-sites test column shows the number of characters shorter in all trees of the specified analysis than in all trees satisfying the Ponder and Lindberg topology for that dataset, followed by the number of characters shorter in all Ponder and Lindberg trees.
In the 'Normal deviate' column, $*P < 0.05$ and $***P < 0.001$.
Refer to text for details of the analyses conducted.

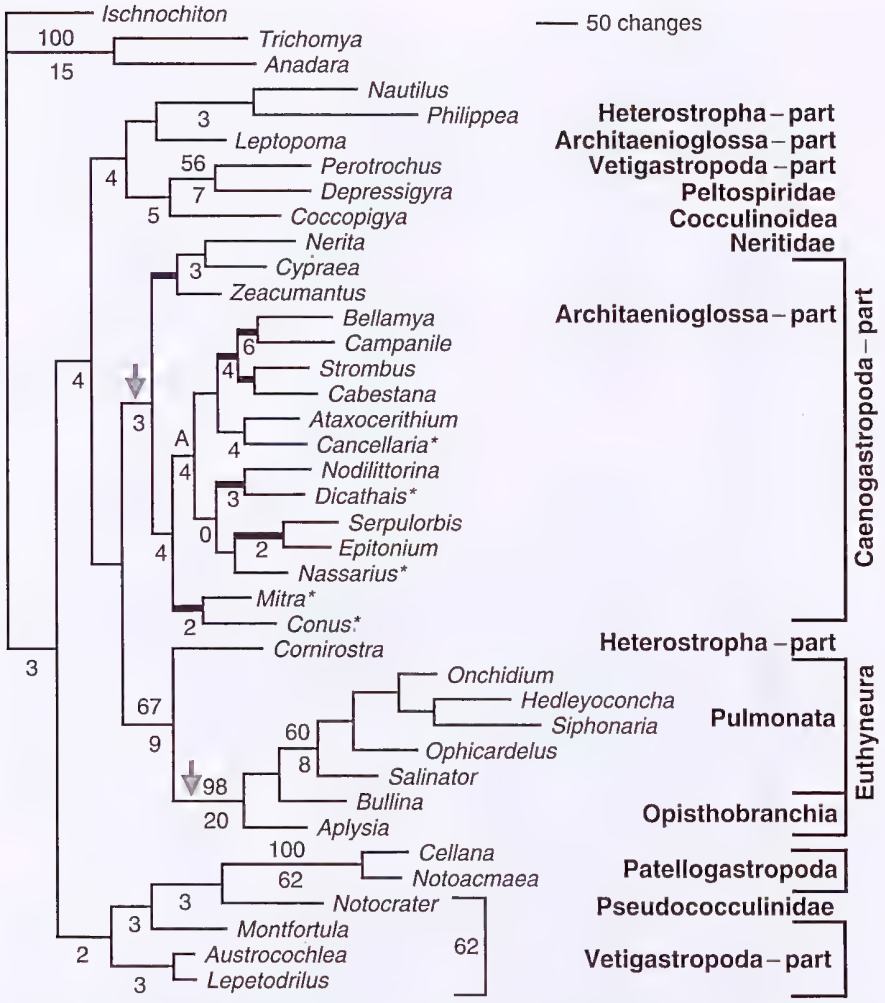

Fig. 2. One of two maximum parsimony trees for all data, excluding areas of uncertain alignment (analysis (*i*)). The topology of both is identical below point A. The arrowheads indicate topological similarities with analysis (*vii*) detailed in the text. Bold branches above A are also seen in the strict consensus of analysis (*i*) and analysis (*vii*). Bootstrap percentages above 50% are shown above a branch. Bremer decay indices are shown below the branch. Branches without figures have indices of 1. Asterisks on genus names indicate membership of Neogastropoda. Note that the brackets on the right-hand side do not necessarily show monophyletic clades.

with *Cornirostra* its sister-group. The clades with puzzling support more than 50% were: (*Nautilus*, *Philippea*) 55%; ((*Trichomya*, *Anadara*) 84%; (*Cellana*, *Notoacmaea*) 51%; ((*Austrocochlea*, *Lepetodrilus*) 68%; *Montfortula*) 61%; ((*Perotrochus*, *Depressigyra*) 65%; *Coccopigya*) 56%; (*Ataxocerithium*, *Cancellaria*) 60%; Pulmonata 52%; Ophisthobranchia (here *Aplysia*, *Bullina*) 69%; (Euthyneura plus *Cornirostra*) 60%.

The topology of the Bayesian analysis (*xi*) with *Ischnochiton* as the outgroup is broadly similar to the results of other analyses, albeit with many instances of high 'posterior probabilities' of clades that are unexpected on morphological grounds but that are shown

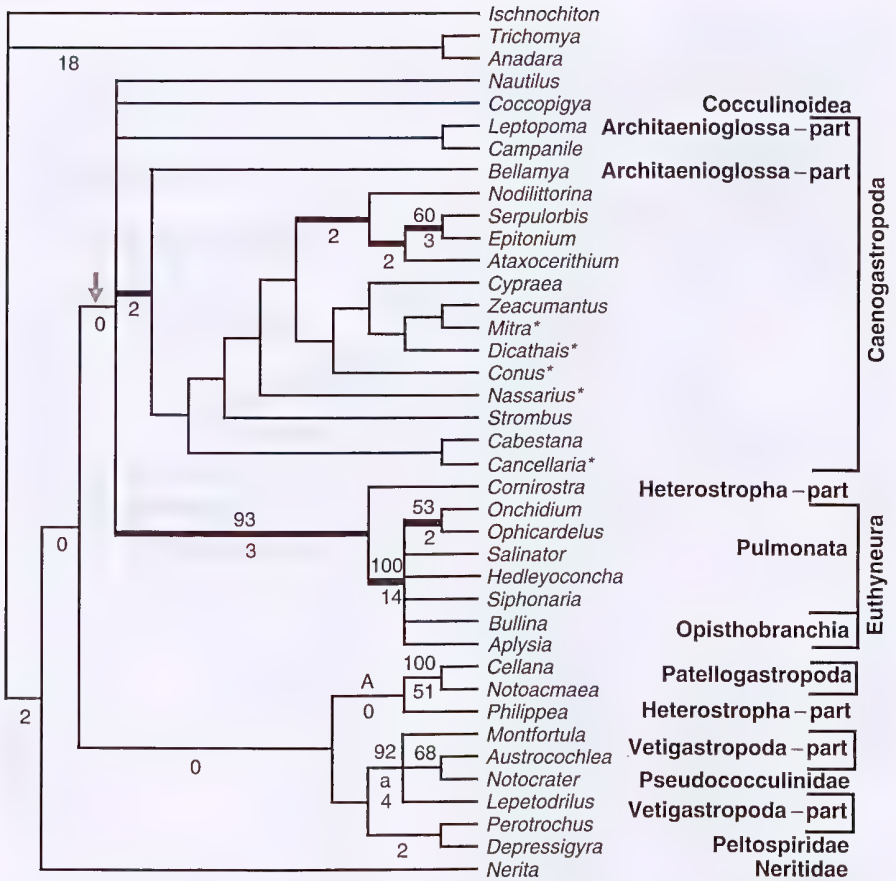

Fig. 3. The strict consensus tree for analysis (*ii*), all data excluding areas of uncertain alignment and third positions. Bootstrap percentages above 50% are shown above a branch. Bremer decay indices are shown below the branch. Branches without figures have indices of 1. Moving branch A to branch a makes the topology of this analysis identical to that for analysis (*viii*) at points below the arrowhead. All Euthyneura except *Siphomaria* are included in a clade with bootstrap support of 54%, although not found in the maximum parsimony tree. Asterisks on genus names indicate membership of Neogastropoda. Note that the brackets on the righ-hand side do not necessarily show monophyletic clades.

in parsimony or maximum likelihood analyses without great support. For instance, the anomalous pairing of *Nerita* with *Cypraea* has a posterior probability of 0.99; the pairing of *Strombus* with *Nassarius* in a group including most Neogastropods has a probability of 0.70 and the pairing of the remaining neogastropod *Cancellaria* with *Ataxocerithium* has a probability of 0.99.

Patellogastropoda was monophyletic with high bootstrap support in all parsimony analyses, but was shown in a basal position only in analysis (*vi*) (Fig. 5). In other analyses, the group was included in a clade with some vetigastropods and *Notocrater* (analyses (*i*), (*vii*); Fig. 2), with this group plus *Depressigyra* (analysis (*x*)), paired with *Philippea* (analysis (*iii*)) as a sister-group to a clade comprising other heterobranchs, caenogastropods, *Coccopigya*, *Nerita* and *Nautilus* (Fig. 4); or paired as sisters with *Philippea* (analyses (*ii*), (*v*); Fig. 3), *Nautilus* (analysis (*iv*)) or *Notocrater*, *Montfortula*,

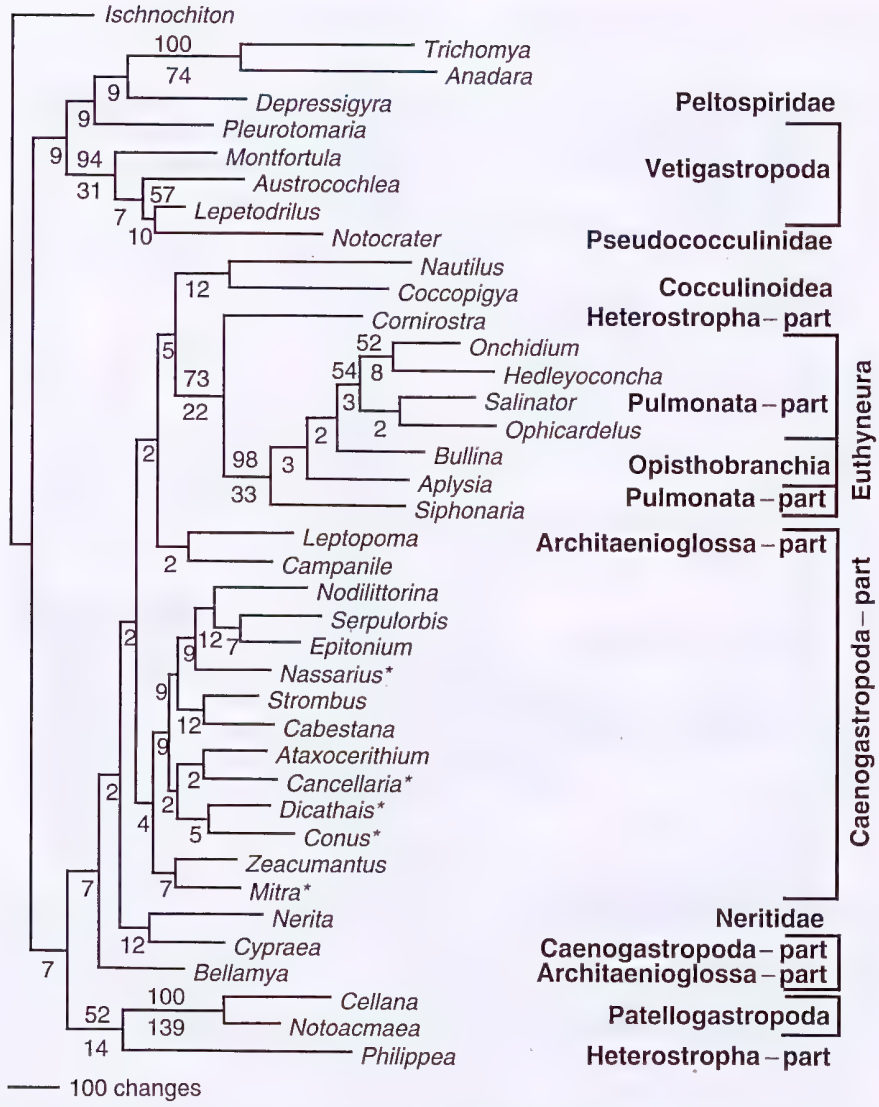

Fig. 4. One of two maximum parsimony trees for all data, excluding areas of uncertain alignment with transition/transversion weighting (analysis (*iii*)). Bootstrap percentages above 50% are shown above a branch. Asterisks on genus names indicate membership of Neogastropoda. Note that the brackets on the right-hand side do not necessarily show monophyletic clades.

Austrocochlea and *Lepetodrilus* (analysis (*viii*)) within a grouping of all vetigastropods and hot-vent taxa except *Coccopigya*. In no case did the pairing of Patellogastropoda as sister-groups with any other taxon receive bootstrap support greater than 50%. In analysis (*xi*), Patellogastropoda is a sister-group to the grouping of all vetigastropods and hot-vent taxa except *Coccopigya* with a posterior probability of 0.52. Imposing the constraint that Eogastropoda and Orthogastropoda were monophyletic sister-groups required 32 more steps ($P \approx 0.11$ using the winning-sites test).

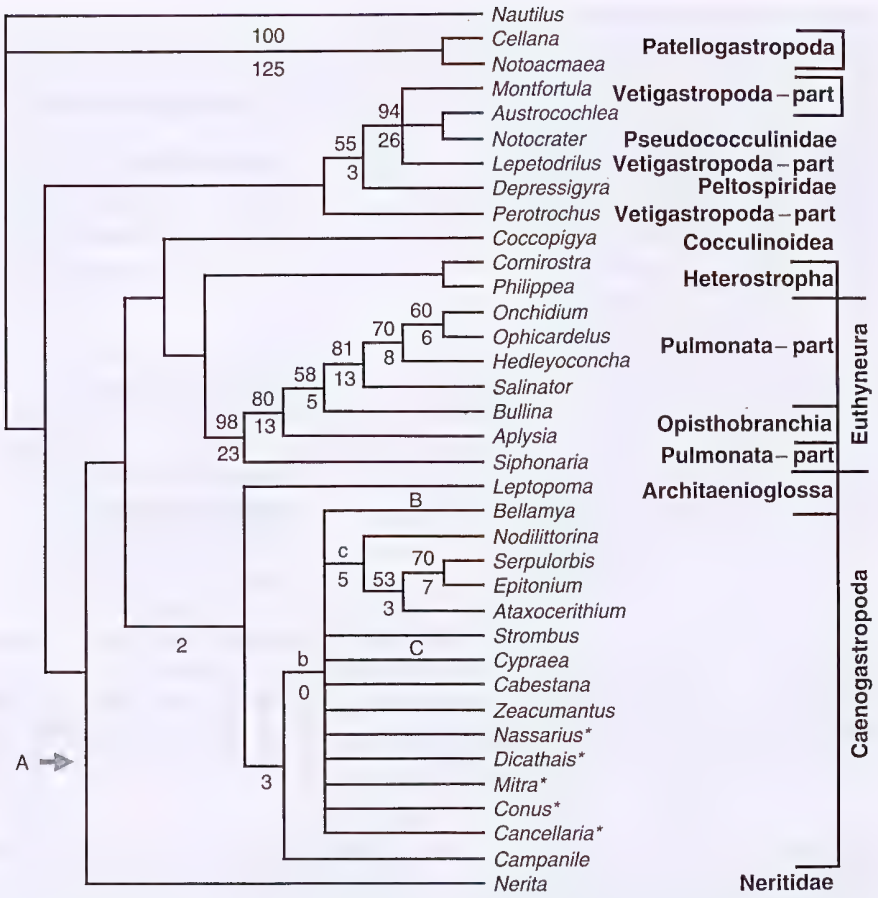

Fig. 5. The strict consensus tree for analysis (*vi*), all data excluding areas of uncertain alignment and third positions, with *Nautilus* as the only outgroup and transition/transversion weighting. Bootstrap percentages above 50% are shown above a branch. Bremer decay indices are shown below the branch. Branches without figures have indices of 1. 'A' indicates the root when the other outgroup taxa are added (analysis (*iv*)). The other differences between the strict consensus trees of these two analyses is that branch B moves to b and branch C to c in analysis (*iv*). Asterisks on genus names indicate membership of Neogastropoda. Note that the brackets on the right-hand side do not necessarily show monophyletic clades.

Cocculiniformia was never monophyletic in our analyses. *Coccopigya* was a sister-group to Euthyneura plus *Cornirostra* in analyses (*ii*), (*iv*), (*vi*), (*viii*), (*ix*), (*x*) and (*xi*), with significant bootstrap support in analyses (*ii*) and (*viii*) and a posterior probability of 100 in analysis (*xi*). It was a sister-group to *Nautilus* in analysis (*iii*) and *Perotrochus* plus *Depressigyra* in analysis (*i*) and analysis (*vii*) and formed one member of a basal trichotomy with *Nautilus* in analysis (*v*). *Notocrater* was always closely associated with a group of Vetigastropoda (*Montfortula*, *Austrocochlea* and *Lepetodrilus*). This group of four taxa was monophyletic with high support in all analyses except analyses (*i*) and (*vii*), where it also included the Patellogastropoda (as a sister-group to *Notocrater*). Even in analyses (*i*) and (*vii*), although the group of four was not shown in maximum parsimony trees, it received

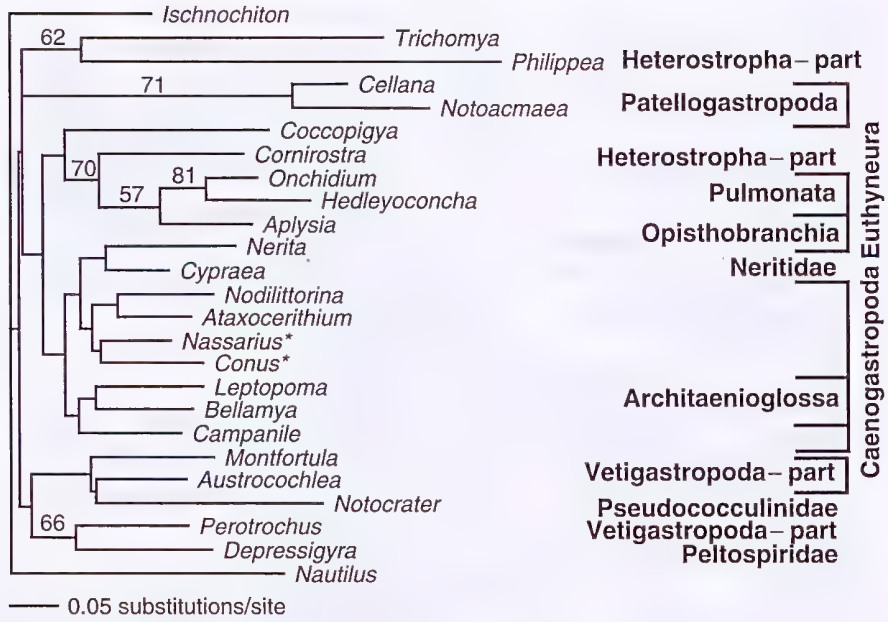

Fig. 6. Maximum likelihood tree of a reduced taxon set, including all data, excluding areas of uncertain alignment. Asterisks on species names indicate membership of Neogastropoda. The figures above branches indicate puzzling support of more than 50%: *Nodilittorina* plus *Nassarius* (54%), *Ataxocerithium* plus *Conus* (60%) and *Montfortula* plus *Austrocochlea* (64%) received puzzling support more than 50%, despite not appearing as clades in the likelihood tree. Note that the brackets on the right-hand side do not necessarily show monophyletic clades.

bootstrap support of more than 60%. Relationships within the group varied, with *Notocrater* being found as a sister-group to each of the three other members in at least one analysis. Imposing the constraint that Cocculiniformia is monophyletic required 18 more steps ($P \approx 0.10$ using the winning-sites test).

Euthyneura was monophyletic in all analyses with high bootstrap support (Figs 2–6; Table 2). Pulmonata and Opisthobranchia were both monophyletic only in analyses (*vii*), (*x*) and (*xi*), here with high posterior probabilities for each clade. In some other analyses (*i* and *ii*), Opisthobranchia was paraphyletic with respect to Pulmonata, but the groups were intermingled in analyses (*iii*), (*iv*), (*v*), (*vi*) and (*vii*), with bootstrap support for a clade of all Euthyneura except *Siphonaria* in analysis (*iv*) (85%) and analysis (*vi*) (80%).

In all analyses, *Cornirostra* was closely associated with Euthyneura, being shown with high bootstrap support or posterior probability as the sister-group to this clade except for analyses (*iv*) and (*vi*). In these analyses, *Cornirostra* was paired with *Philippea* as a sister-group to Euthyneura to form a monophyletic Heterobranchia, with bootstrap support of 67% in analysis (*iv*). Generally, Heterobranchia was disrupted by the association (not bootstrap supported) of *Philippea* with other taxa: *Nautilus* in analyses (*i*) and (*x*); and Patellogastropoda in analyses (*ii*), (*iii*) and (*v*). Imposing the constraint that Heterobranchia is monophyletic required 23 more steps ($P \approx 0.22$ using the winning-sites test).

The genetic divergence of the Heterobranchia as measured by the distance of terminals from the root in maximum parsimony analyses is striking, although less pronounced in likelihood analysis.

In each analysis except analyses (*v*) and (*vii*), the great majority of the Caenogastropoda and Heterobranchia grouped to form a recognisable but weakly supported 'apogastropodan clade'. Examples of the exclusion of *Philippea* from this are listed above. *Lepotopoma* was excluded in analyses (*i*) and (*vii*). Unexpectedly included taxa are *Nerita* in analysis (*i*), (*iv*), (*viii*), (*ix*) and (*x*), *Nautilus* and *Coccopigya* in analyses (*ii*) and (*iii*), and *Coccopigya* in analysis (*vi*). In analysis (*v*), Euthyneura plus *Cornirostra* was a sister-group to all other gastropods except *Coccopigya*. In analysis (*vii*), five unexpected taxa (*Nautilus*, *Nerita*, *Pterotrochus*, *Depressigyra* and *Coccopigya*) disrupt the 'apogastropod' lineage. None of the unexpected inclusions or exclusions had bootstrap support greater than 50%.

Discussion

The present analyses used data from six gene segments from four loci to address four of the major differences between morphological (Haszprunar 1988*a*; Ponder and Lindberg 1997) and molecular (Tillier *et al.* 1992, 1994; Rosenberg *et al.* 1997; McArthur and Koop 1999; Colgan *et al.* 2000; Harasewych and McArthur 2000) understanding of gastropod relationships. These were: (*1*) the position of Patellogastropoda; (*2*) the relationships of members of Cocculiniformia; (*3*) the relationships of members of Neritopsina; and (*4*) the monophyly of Heterobranchia.

From the molecular perspective, the division of Gastropoda into Eogastropoda and Orthogastropoda remains an open question, despite the addition of more data in the present paper. The position of Patellogastropoda varies in present analyses as in previous molecular investigations (Rosenberg *et al.* 1997; McArthur and Koop 1999; Colgan *et al.* 2000). Long-branch length attraction (Felsenstein 1978; Lyons-Weiler and Hoelzer 1997; Siddall and Whiting 1999; Stiller and Hall 1999; Philippe and Germot 2000) may be a possible explanation for the pairing of Patellogastropoda with groups that would be unexpected on morphological grounds. Morphological support for the eogastropod/orthogastropod division is strong, but not incontestable. Character states supporting the division of Gastropoda into Eogastropoda and Orthogastropoda should be synapomorphic in the latter group and plesiomorphic or having an autapomorphy not derived from the state in Orthogastropoda in Patellogastropoda. The potential number of such characteristics is impressive. Fifteen changed state between the nodes uniting all gastropods and all orthogastropods in the Ponder and Lindberg (1997) topology. However, five have consistency indices less than 0.4 and one (adult operculum) is not applicable to Patellogastropoda, although they possess a larval operculum. Among the other nine characteristics, only three (18, the presence of a hypobranchial gland; 60, the bending plane of the radula; and 98, statocyst position) have possibly derived states in all major orthogastropod clades. In the remaining six characteristics, some or most Vetigastropoda share the same derived state as Patellogastropoda or have the symplesiomorphic state for Gastropoda.

The situation with some characteristics is ambiguous; for example, with the hypobranchial gland. Sasaki (1998; his character 25) confirmed that a hypobranchial gland is absent in patellogastropods and noted its absence in *Nautilus* (although the nidamental glands may be homologous; Salvini-Plawen 1990). Sasaki (1998) also stated that the gland is absent in Fissurellidae on the basis of his own observations and Neomphalidae (*fide* McLean 1981), although it was recorded as present in these taxa by Ponder and Lindberg (1997). Fretter and Graham (1962) recorded a hypobranchial gland in *Diodora* and implied its presence in *Emarginula* (both fissurellids). Haszprunar (1989*b*) observed a small hypobranchial gland in males of *Pseudorimula* (a fissurellid) but not in females. Israelsson

(1998) recorded a hypobranchial gland in *Pachydermia* (related to *Neomphalus*) and one has also been recorded in *Melanodrymia* (Haszprunar 1989*a*), but the presence or absence of one is not noted in *Neomphalus* by Fretter *et al.* (1981). Sasaki (1998) bases his statement regarding the absence of a hypobranchial gland on McLean (1981), who says that a thick folded gland, as seen in haliotids and trochids, is absent but that there are '...scattered subepithelial gland cells ... comparable to .... the Fissurellidae in which gland cells are present in the mantle skirt but do not form a discrete organ with a folded surface'. Given the presence of an undisputed gland in closely related taxa, what is present in *Neomphalus* is certainly a reduced hypobranchial gland, similar reductions being seen in many other gastropods, even within genera. These observations do not discount the possibility that the hypobranchial glands are secondarily absent in patellogastropods. They are absent in Scaphopoda, and possibly Cephalopoda, and a possibly homologous gland is present in Monoplacophora (Lemche and Wingstrand 1959; Wingstrand 1985; Haszprunar 1997).

One of the characteristics supporting the monophyly of the orthogastropods, the flexoglossage condition of the radula (Haszprunar 1988*a*; Salvini-Plawen 1988; Ponder and Lindberg 1997), is now thought to be plesiomorphic, owing to some lateral bending of the radula being found in chitons (Guralnick and Smith 1999). Guralnick and Smith (1999) suggest that the stereoglossate condition of the radula in patellogastropods is secondary. The radular stroke of living monoplacophorans has not been examined, so the condition of their radula can only be inferred. However, Guralnick and Smith (1999) argue that it is also probably flexoglossate with the structure of the radula most like that of lepetid patellogastropods. Available data also suggest a flexoglossate condition in cephalopods, scaphopods and in 'aplacophorans' (Guralnick and Smith 1999). Thus, on the basis of these findings, the stereoglossate condition appears to be an autapomorphy of the patellogastropods. There are, however, some plesiomorphic states retained in the patellogastropod radula that are not found in other gastropods (Guralnick and Smith 1999).

Since the publication of Ponder and Lindberg (1997), two new datasets add additional weight to the basal position of the patellogastropods. These relate to the buccal cartilages and the fine structure of the cephalic tentacles.

Only the number of buccal cartilages present in the odontophore was scored by Ponder and Lindberg (1997). Sasaki (1998) and Guralnick and Smith (1999) have attempted to homologise the cartilages. Guralnick and Smith (1999) used position and shape as a primary means of tracking the evolution of the buccal cartilages. They argue that a medial pair of cartilages is plesiomorphic for Mollusca, as also (probably, therefore being secondarily absent in 'aplacophorans') are the dorsolateral (= anterolateral of Sasaki (1998)) cartilages. More likely, assuming 'aplacophorans' are basal (e.g. Haszprunar 2000), the cartilages are probably synapomorphic of Testaria (*sensu* Haszprunar 2000). Whereas Sasaki (1998) considered these latter cartilages to be autapomorphies of patellogastropods, Guralnick and Smith (1999) argued that they were homologues of the dorsolateral cartilages of chitons and monoplacophorans. In these latter taxa, the two pairs of cartilages are attached by a connective tissue sheath, the space between being the hollow vesicles seen in those groups. Dorsolateral cartilages are absent in all Apogastropoda. A pair of dorsal cartilages is found in chitons and these are absent in modern Monoplacophora, but present in some patellogastropods. In addition, there are two pairs of posterior cartilages in chitons and the patellid patellogastropods (absent, presumably lost, in some of the more modified patellogastropods and in living Monoplacophora; Guralnick and Smith 1999). A single posterior pair is found in some vetigastropods and neritopsines. In patellogastropods, the subradular membrane is not associated with the medial cartilages

as it is in other gastropods, but is, instead, associated with the plesiomorphic dorsolateral (and dorsal cartilages when present), lying well above the medial cartilages.

Künz and Haszprunar (2001) showed that the fine structure of the cephalic tentacles of patellogastropods differs significantly from that of vetigastropods and neritopsines and that they share ciliary features observed in bivalves and 'aplacophorans'. A similar configuration (stiff cilia with a more or less homogeneous pattern of microtubules) is unknown in most other gastropods, although somewhat similar cilia are known from the tentacles of the pulmonate *Lymnaea* (Emery 1992). In addition, the ciliary tufts of patellogastropods have several ciliary types, whereas in the other two groups the ciliary morphology is much more uniform. Further, patellogastropods, vetigastropods and neritopsines all show differences in their sensory elements, supporting and mucous cells.

Other recent datasets that are less well resolved, but also appear to show that the patellogastropods are distinct from the vetigastropods and other gastropods, include larval musculature and the development of adult muscles (Wanninger *et al.* 1999) and sperm ultrastructure (e.g. Hodgson and Morton 1998). In other characteristics (e.g. cleavage pattern (van den Bigelaar and Haszprunar 1996), larval morphology and ciliation (Hadfield *et al.* 1997)), the patellogastropods and vetigastropods share assumed plesiomorphic conditions.

A significant problem for the patellogastropod ancestors being the sister-group to the orthogastropods is the lack of undoubted patellogastropods or obvious coiled ancestors in the early fossil record. The oldest undoubted patellogastropod has been confirmed recently (on the basis of shell structure) from the Triassic (Hedegaard *et al.* 1997). Recognition of such, probably coiled, ancestors will be difficult, but Wagner (2002) very tentatively suggests 'euomphalinaes' as candidates. If this was the case, the split in the two main gastropod lineages occurred in the Late Cambrian, around 510 million years ago.

Cocculiniformia are not monophyletic in our analyses. *Notocrater* (Pseudocculinidae) is strongly associated with Vetigastropoda, as found by Ponder and Lindberg (1997). *Coccopigya* is variously associated in derived positions (e.g. with Heterobranchia or *Depressigyra* and *Perotrochus*). The pairing with *Nerita* at the base of Orthogastropoda observed by Ponder and Lindberg (1997) is not found in any of our analyses. There are no morphological characteristics directly suggesting that Cocculinidae and Heterobranchia are sister-groups, although the sinistral protoconch coiling found in all members of the latter group has its analogue in at least some Cocculinidae. The pairing of *Coccopigya* with two other deep-sea taxa (*Depressigyra* and *Perotrochus*) appears to be coincidental because *Notocrater* and *Lepetodrilus* are also found in this environment.

Heterobranchia is rarely monophyletic in our analyses, owing to the variable placement of *Philippea*. Euthyneura is monophyletic in all analyses. Within Euthyneura, Opisthobranchia and Pulmonata are rarely monophyletic, concurring with Dayrat *et al.* (2001). They found that Opisthobranchia is paraphyletic with respect to Pulmonata, albeit that the nodes suggesting this observation had low bootstrap support.

The clade comprising Euthyneura plus *Cornirostra* is strongly supported in our analyses, supporting the suggestion of Haszprunar (1988*a*) that Valvatoidea is closer to Euthyneura than is Architectonicoidea. Confirmation of this will require data from other members of the family because *Philippea* is undoubtedly highly autapomorphic and its placement appears to depend on long-branch attraction. Questions of monophyly or paraphyly of Heterostropha, which includes the well-established families Omalogyridae, Pyramidellidae, Valvatoidea, Architectonicoidea and Rissoellidae, as well as a number of recently created Recent and fossil families, will be a fruitful area for further research.

The relatively large genetic differentiation of Heterobranchia in maximum parsimony trees suggests that the clade has a long evolutionary history, particularly if substitution rates are even remotely clocklike. The genetic distinction of Heterobranchia is emphasised by mitochondrial DNA genome organisation. In studied Euthyneura (for references, see Kurabayashi and Ueshima 2000*a*), this is radically different to the arrangement in the caenogastropod *Littorina* (Widling *et al.* 1999) but similar to that of *Omalogyra* (Kurabayashi and Ueshima 2000*b*). Other gene order work on opisthobranchs (Grande 2001; Medina *et al.* 2001) has yet to be reported in full. To date, the gene order data are based on an extremely small sampling and whether or not *Littorina* is typical of caenogastropods is unknown. For example, Collins *et al.* (2001) and Rawlings *et al.* (2001) report a major gene order rearrangement within the caenogastropod Vermetidae. Sperm structure (Healy 1993) also supports the monophyly of heterobranchs as a whole and Euthyneura. The earliest undoubted heterobranch fossils date from the early Devonian (390–408 million years ago; Frýda and Blodgett 2001), although some taxa included in the subulitoideans are likely heterobranchs and this grouping extends into the Ordovician (Nützel *et al.* 2000).

The affinities of the Heterobranchia (excepting *Philippea*) are with the Caenogastropoda in a recognisably 'apogastropodan' group (Salvini-Plawen and Haszprunar 1987; Haszprunar 1988*a*; as extended by Ponder and Lindberg 1997), although some taxa are anomalously included or excluded in some analyses. This contrasts with Colgan *et al.* (2000), where the 'apogastropod' group also had unexpected inclusions (*Nerita* and *Nautilus*) and Caenogastropoda and Heterobranchia were intermixed. Apogastropoda are monophyletic and comprised of monophyletic Caenogastropoda and Heterobranchia in McArthur and Koop (1999) and Harasewych and McArthur (2000), although these studies include fewer taxa from these latter groups.

Caenogastropoda is monophyletic with the exception of the anomalous inclusion of *Nerita* and/or *Nautilus* in some analyses and the exclusion of *Leptopoma* and *Cypraea* in analyses (*i*) and (*vii*). Relationships within Caenogastropoda are not well resolved in the present analyses. In particular, although various sets of two of the five genera of Neogastropoda included in the dataset are found in monophyletic clades in some analyses and four of the five are grouped in the Bayesian analysis (*xi*), this morphologically strongly supported group is not otherwise shown as closely related, as also found by Harasewych *et al.* (1997*b*).

Architaenioglossa comprise a number of superfamilies (previously two, now three) not considered close relatives by Ponder and Lindberg (1997). The taxa included here, *Bellamya* representing Viviparioidea (previously included within what is now considered to be a separate superfamily Ampullarioidea) and *Leptopoma* representing Cyclophoroidea, are monophyletic only in analysis (*ix*) based on maximum likelihood and analysis (*xi*) based on Bayesian likelihood. *Bellamya* is a member of the Caenogastropoda in all our analyses, but the position of *Leptopoma* varies widely although remaining within an apogastropodan group, except when all data are considered (analysis (*i*)), where it is a sister-group to an heterogeneous taxon (Fig. 2). *Campanile* is always associated with *Bellamya* or *Leptopoma*. McArthur and Koop (1999), using partial *28S* rDNA sequences, also found that the architaenioglossans (*Ampullaria* and *Viviparus*) were not monophyletic and, unlike our result, that *Ampullaria* and *Campanile* were sister-taxa. In their analyses, and with most of our analyses, the architaenioglossans lie within the caenogastropods, as suggested by Ponder and Warén (1988) and demonstrated in the morphological analyses of Ponder and Lindberg (1996, 1997). Alternative hypotheses have been produced on the basis of

morphological analyses, notably suggesting that the architaenioglossans are the sister-group to the apogastropods and part of a paraphyletic 'Archaeogastropoda' (Haszprunar 1988*a*) or that they belong to a clade (together with Neritopsina and Neomphaloidea), which is sister-group to the caenogastropods (Barker 2001).

The placement of the Neritopsina (or Neritimorpha) remains uncertain. This group, plus the Cocculiniformia (among our studied taxa), formed a sister-clade to all other Orthogastropoda in Ponder and Lindberg's (1997) preferred topology and in Rosenberg *et al.* (1997), although in this latter analysis the patellogastropod was included within the apogastropods. In the morphological analysis undertaken by Sasaki (1998), the patellogastropods formed the base of the gastropod clade and *Cocculina* appeared within a clade containing *Neomphalus* and the vetigastropods. Neritopsines formed one of four branches in an unresolved Gastropoda in the analysis of Harasewych *et al.* (1997*a*) and one branch of a basal trichotomy in McArthur and Koop (1999: fig. 3) and our analysis (vi). As in analyses (*ii*), (*iv*) and (*viii*), the Neritopsina was a sister-group to all other gastropods in maximum parsimony analyses (excluding one 25 bp insert) of the *18S* rDNA data of Harasewych and McArthur (2000: fig. 2*A*) and their maximum likelihood analysis (fig. 2*C*). When two longer inserts were excluded, Neritopsina was a sister-group to Vetigastropoda, this pair being a sister-group to Apogastropoda (Harasewych and McArthur 2000: fig. 2*B*). *Nerita* was placed within Caenogastropoda in Colgan *et al.* (2000), in accordance with our analyses (*i*), (*v*), (*vii*), (*ix*) and (*xi*) but in contrast with all recent morphological assessments (cf. Bieler 1992). In Barker's (2001) morphological analysis, Neritopsina was the sister-group to a clade consisting of Neomphaloidea + Architaenioglossa. This combination was a sister-group taxon to the caenogastropods. In our analyses (*iii*) and (*vi*), *Nerita* is a sister-group to Apogastropoda, allowing the possibility that larval planktotrophy arose once only in gastropods (cf. Ponder 1991; see also discussion in Ponder and Lindberg 1997: 209–213; and Frýda 2001).

The strict consensus of the maximum parsimony trees from analysis (*vi*) has notable similarities to recent morphological hypotheses. Agreeing with Ponder and Lindberg (1997), Caenogastropoda and Heterobranchia are monophyletic, as is Vetigastropoda, with the predicted inclusion of *Notocrater*. Patellogastropoda is basal, although excluded from Gastropoda.

Although the results of all analyses should be included in discussions of the phylogenetic implications of our data, we give a little more weight, when results differ, to those of analysis (*vi*), where, with *Nautilus* as the only outgroup, third-position data are excluded and transitions and transversions are weighted differently (Fig. 5). Comparison of consistency indices supports the exclusion of data because they are higher in trees including third positions than those excluding them. As judged by the consistency indices, excluding these data reduces the amount of phylogenetic noise. Arguing for differential weighting is the low transition to transversion ratio in the overall data for coding genes *CO1* and *H3*. This ratio increases when third positions are excluded, indicating a substantial degree of saturation. This analysis (as well as some others) has a high probability of homogeneity of phylogenetic inferences from the separate gene data.

When the chiton and bivalves are included, the outgroups are not monophyletic in any analysis. The use of *Nautilus* as the sole outgroup is suggested by the consensus on morphological grounds that Cephalopoda or Monoplacophora are the sister-taxon to Gastropoda (reviewed by Ponder and Lindberg 1997). Although not included in our analysis, scaphopods have recently been shown to be the sister-taxon to the cephalopods (Waller 1998; Haszprunar 2000; Giribet and Wheeler 2002; Wanninger and Haszprunar

2002), in contrast with earlier hypotheses that linked them to the bivalves. This relationship is, however, not apparent in the analysis of Rosenberg *et al.* (1997).

Unfortunately, despite considerable advancement in our knowledge of Palaeozoic fossils in the past decade, the origins of the major gastropod groups remain obscure, although all should have differentiated by the early Ordovician shortly after gastropods evolved (Wagner 2001, 2002). Whereas the considerable extinctions that have occurred during gastropod evolution may account for some of the long branch attraction issues encountered (especially between the patellogastropods and the remainder of the gastropods), breaking down some of the long branches encountered in this dataset by the addition of more taxa (Graybeal 1998) may be possible.

Despite more molecular data having been incorporated in these present analyses, some major aspects of gastropod phylogeny remain equivocal. Additional genes, gene order data and more refined morphological data will be required to resolve many of these issues, as well as better data on Palaeozoic gastropods.

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Reassessment of Australia's oldest freshwater snail, *Viviparus* (?) *albascopularis* Etheridge, 1902 (Mollusca: Gastropoda: Viviparidae), from the Lower Cretaceous (Aptian, Wallumbilla Formation) of White Cliffs, New South Wales

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Abstract

Viviparus (?) *albascopularis* Etheridge, 1902 is Australia's oldest documented fossil freshwater gastropod. The taxon was established on the basis of a single opalised shell from the Lower Cretaceous (Aptian) marine deposits of the Wallumbilla Formation (Doncaster Member) at White Cliffs, New South Wales. Reassessment indicates that original placement in the caenogastropod family Viviparidae is justified; however, the specimen is reassigned to the endemic Australian genus *Notopala* Cotton, 1935 on the basis of shell morphology and close morphometric similarity to extant species. Implications for the origins and current distribution of Australian viviparid taxa are discussed.

Introduction

The fossil record of Australian Cretaceous non-marine gastropods is depauperate and very poorly known. Nearly all the currently identified material has been recovered from the middle–upper Albian (Lower Cretaceous) fluviatile–lacustrine deposits of the Griman Ck Formation at Lightning Ridge in north-western New South Wales (NSW). This assemblage comprises primarily viviparids, although numerous other groups, including thiarids (Hamilton-Bruce *et al.* in press), succinids, camaenids (currently under study) and amphibolids (B. J. Smith, R. J. Hamilton-Bruce and B. P. Kear, unpublished data) are also present (Dettman *et al.* 1992; Smith 1999; Hamilton-Bruce *et al.* 2002). The only other documented Australian Cretaceous non-marine gastropod fossil is a single opalised shell (AM F17456) from the Aptian (Lower Cretaceous) marine sediments of the Wallumbilla Formation (Doncaster Member) at White Cliffs, NSW (Burton and Mason 1998: see figs 1,2 for detailed geological and locality maps of the area). This specimen was described (from a private collection belonging to a Mr H. Y. L. Brown of Adelaide, later acquired by the Australian Museum) by Etheridge (1902) and tentatively assigned to *Viviparus* (?) *albascopularis*, recognising similarity to members of the currently extant caenogastropod family Viviparidae. The present paper provides a revised description of the holotype (AM F17456) and only known specimen of *V.* (?) *albascopularis* Etheridge and reinterprets its taxonomic placement. Implications for the origins and distribution of Australian viviparid taxa are discussed.

The Lower Cretaceous (Aptian) opal-bearing deposits of White Cliffs have long been known as a productive locality for fossils. Anderson (1892) briefly remarked on the presence of mollusc remains, crinoids and wood. Jaquet (1893) recorded belemnitid

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cephalopods and Devonian invertebrate fossils preserved as impressions in large erratic clasts. These were interpreted as a product of reworking from underlying Palaeozoic conglomerates. Etheridge (1897, 1902, 1904) reported the occurrence of bivalves, ammonites, naticid gastropods, plesiosaurs and ichthyosaurs. More recent studies by White (1926), Molnar (1980, 1991) and Kemp (1991) have also identified lungfish and dinosaur remains.

Viviparus (?) *albascopularis* Etheridge is currently the oldest known Australian fossil freshwater gastropod and one of the earliest members of the Viviparidae. Today, this cosmopolitan family comprises various taxa, characterised by medium–large-sized turbiniform shells, possessing a rounded body whorl, moderately high and pointed spire, wide, round aperture and a concentric, horny operculum (Smith 1992). Within Australia, the distribution of the group is limited to a few species occurring in the large drainage basins that span much of the arid centre, northern tropical and coastal regions.

The fossil record for Viviparidae is known from the Jurassic–Recent (*Viviparus* Montfort, 1810), with a tentative report based on an internal shell mold (?*Bernicia* Cox, 1927), possibly of marine origin, from the Early Carboniferous of England (Brookes-Knight *et al.* 1960). The group's Australian record is very sparsely documented. Cotton (1935) described a species of *Notopala* Cotton, 1935 (*N. wanjacalda*) from upper Pleistocene sediments along the Murray River, near Sunnyside, South Australia (SA), and noted a second taxon (*Notopala* sp.) from the same deposit, which showed strong similarity to the extant *N. hanleyi* (von Frauenfeld, 1862). Dettman *et al.* (1992) reported viviparid snail shells from the Lower Cretaceous (middle-upper Albian) deposits of Lightning Ridge, NSW, as did Smith (1999), who also recorded representatives of the Naticidae, Thiaridae and Ellobiidae. Recently, Hamilton-Bruce *et al.* (2002) described a new genus (*Albianopalin*) and two new species of viviparid from Lightning Ridge, as well as indeterminate material attributable to the currently extant endemic Australian taxon *Notopala*. Other Australian non-marine gastropod fossils (all of Tertiary age) have been documented by Chapman (1937), McMichael (1968), Archer *et al.* (1994), Arena (1997) and Pledge *et al.* (2002). Occurrences from elsewhere in Australasia are rare, particularly in Cretaceous sediments. Some of the few examples include viviparids (genera uncertain) and thiarids (?*Melanoides* Olivier, 1804) from the Cenomanian–?Santonian (Upper Cretaceous) of New Zealand (Henderson *et al.* 2000) and possible thiarids (*Pyrgulifera* Meek, 1871) from both the Campanian–lower Maastrichtian (Upper Cretaceous) of the Chatham Islands (Stilwell 1998) and ?Campanian of New Caledonia (Henderson *et al.* 2000).

Material and methods

Material registered as the holotype of *V.* (?) *albascopularis* Etheridge includes a single shell with broken aperture margin and protoconch (AM F17456), preserved entirely in potch (non-precious or common opal). The specimen is derived from an unknown mine locality in the opal-bearing deposits of White Cliffs near Wilcannia in north-western NSW. The lithostratigraphic nomenclature for Lower Cretaceous rocks of the White Cliffs area was recently discussed by Burton and Mason (1998), who placed them within the Doncaster Member of the Wallumbilla Formation (Eromanga Basin), a unit of Aptian–middle Albian (115–approximately 100 million years ago; *sensu* Lowrie *et al.* 1980) age. However, the White Cliffs opal-bearing sediments are regarded as representing only the lower Aptian section of the Doncaster Member and comprise predominantly sandy/silty claystone and fine-grained sandstones deposited in a near-shore coastal marine setting (Burger 1988; Burton and Mason 1998). Determinations of palaeolatitude place the White Cliffs area as high as 70°S during the Early Cretaceous (Embleton 1984). Palaeoclimatic indicators for the region also suggest predominantly cool, strongly seasonal conditions with winter freezing (Frakes and Francis 1988, 1990; Sheard 1990; Frakes *et al.* 1995; De Lurio and Frakes 1999; Henderson *et al.* 2000). Estimates of sea level isotopic palaeotemperatures in the south-western section of the

Eromanga Basin have yielded averages as low as 12.2°C (Stevens and Clayton 1971; Dettman *et al.* 1992). However, Selwood *et al.* (1994) reported revised isotopic data supporting much cooler ocean temperatures during the Early Cretaceous. Indeed, Pirrie *et al.* (1995) indicated palaeotemperatures of around 10°C based on Early Albian belemnites from the Carnarvon Basin, Western Australia (situated at approximately 45° palaeolatitude during the Cretaceous). In contrast, Huber *et al.* (1995) and Huber and Hoddell (1996) argued that minimal pole-to-equator thermal gradients existed during much of the Middle–Late Cretaceous. This was also discussed by Henderson *et al.* (2000), who noted that although palaeotemperatures at 70°–80° latitude would certainly have been more equitable than they are today, evidence such as the distinct growth-banding in Australian Cretaceous wood (Dettman *et al.* 1992), and the presence of potentially ice-rafted quartzite/porphyritic boulders (Frakes and Francis 1988, 1990; Frakes *et al.* 1995) and glendonites (crystal aggregates pseudomorphing the calcium carbonate hexahydrate mineral ikaite; Sheard 1990; DeLurio and Frakes 1999) attests to the strong seasonality and winter freezing along the inboard extremity of the Australian epicontinental seaway during the Aptian.

Shell diameters were measured using the method of Boycott (1928), defined as '... the greatest dimension that can be found starting with the edge of the lip to a point on the opposite side of the shell on the last whorl'. Shell measurements were made to the nearest 0.05 mm using dial calipers.

The Australian Museum, Sydney is abbreviated as AM throughout.

Systematics

Class **GASTROPODA**

Superorder **CAENOGASTROPODA**

Superfamily **VIVIPARIOIDEA**

Family **VIVIPARIDAE** Gray, 1847

Diagnosis

Medium to large dextral, turbiniform shells with rounded body whorl, spire moderately high and pointed, aperture wide and round to distinctly lunate–ovate, lirae present or absent and horny, concentric operculum.

Remarks

The above diagnosis follows Smith (1992), modified to accommodate the presence of a distinctly lunate–ovate aperture and spiral lirae on the last body whorl of the holotype specimen, AM F17456. Viviparid snails are, as their name suggests, live bearing and are found in both lotic and lentic systems throughout the world (Browne 1978). Within Australia, the family is currently represented by the extant native genera *Notopala*, *Larina* Adams, 1851 and *Centrapala* Cotton, 1935 (see Smith 1992) and the introduced *Bellamya heudei guangdungensis* (Kobelt, 1906), an Asian species now recorded in the wild in NSW (Shea 1994).

Australian endemic viviparids have undergone extensive taxonomic revision in recent years (the most inclusive analysis currently being undertaken by W. Ponder of the Australian Museum, personal communication, 2002), with better understanding of intraspecific shell variation and morphometric data resulting in a substantial reduction in the number of accepted species (see Sheldon and Walker 1993). However, many of the key characteristics used to determine interrelationships, such as shell colour and form of the operculum, are usually lost in fossil material; therefore, only structural features of the shell (see below) can be used to assign them to taxa.

Genus *Notopala* Cotton, 1935

Notopala Cotton, 1935: 339. Type species (by original designation): *Paludina hanleyi* von Frauenfeld, 1864.

Diagnosis

Shell dextral, globose–conic, subumbilicate, up to 5 whorls, ventricose to angulate below the periphery; spiral lirae present on periostracum of last body whorl in some taxa; aperture large and subovate to lunate–ovate, aperture size equal to or greater than height of spire, operculum corneous (unknown in fossil taxa).

Remarks

Despite the recovery of AM F17456 from a deposit of marine origin, its shell morphology fits within the currently accepted diagnosis of *Notopala* and Viviparidae with only slight modification (the presence of a distinctly lunate–ovate aperture and calcified spiral lirae on the last body whorl). To justify placement within the family and to establish a basis for both generic reassignment and comparison with extant species, we have applied parts of the morphometric data gathered by Sheldon and Walker (1993). The histogram in Fig. 1 is based on measurements of shell characteristics for each of the living Australian species of *Notopala* (derived from Sheldon and Walker 1993) and illustrates the morphometric similarity of AM F17456, redescribed herein, to currently existing members of the genus.

Notopala albascopularis (Etheridge, 1902)

(Fig. 2*A–D*)

Viviparus (?) *albascopularis* Etheridge, 1902: 43; pl. VII, figs 8,9.

Material examined

Holotype. AM F17456. Type locality and horizon: White Cliffs Opal Field (exact mining claim locality unknown), near Wilcannia, north-western NSW. The deposits form part of the Doncaster Member of the Wallumbilla Formation (Rolling Downs Group), Eromanga Basin, and are of Aptian age (see Burton and Mason 1998). This corresponds to the *Cyclosporites hughesii*–lower-most *Crybelosporites striatus* spore-pollen Zones and *Odontochitina operculata–Diconodinium davidii* dinoflagellate zones of Helby *et al.* (1987).

Diagnosis

With the features of the genus; irregular, pustular lirae present on the last body whorl; aperture somewhat poorly preserved but distinctly lunate–ovate and markedly adapically situated.

Description

Shell (Fig. 2*A–C*) dextral, turbiniform, very slightly carinate, subglobose, 21.65 mm high, 20.05 mm maximum diameter, 13.55 mm aperture length, 11.5 mm aperture width, 8.1 mm spire length. Teleconch four complete whorls and broken parts indicating further whorls, probably up to five. Whorls impressed. Relatively evenly spaced fine spiral prosocline growth lines on lower three whorls. Aperture large (13.55 mm high), slightly subangular, distinctly ovate–lunate, markedly adapically situated. Opal replacement of original shell material indicates 12 or more irregular, pustular lirae on the last body whorl (Fig. 2*D*).

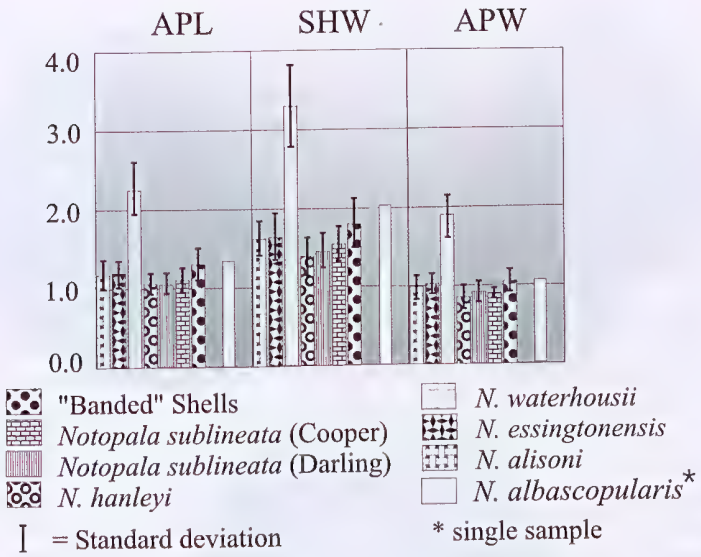

Fig. 1. Histogram showing results of morphometric analysis. Taxa include living species of *Notopala* (measurements modified from Sheldon and Walker 1993) and *N. albascopularis* (Etheridge, 1902), AM 17456. APL, aperture length; APW, aperture width; SHW, shell width.

Remarks

Holotype unique. The presence of irregular, pustular lirae on the last body whorl and a distinctly lunate–ovate, markedly adapically situated aperture separates *N. albascopularis* (Etheridge, 1902) from other species of *Notopala*. The generic reassignment of the holotype specimen is highly significant because previously none of the currently living native Australian viviparid genera was known from deposits older than middle–upper Albian (upper-most Lower Cretaceous). This temporal range is now extended back to the Aptian.

Discussion

The fossil record of Australian non-marine gastropods is very sparsely documented, with the majority of existing reports describing material of Tertiary to Holocene age. Mesozoic specimens have, to date, only been identified from middle to upper Albian (Lower Cretaceous) sediments of the Griman Ck Formation at Lightning Ridge in northern NSW (Dettman *et al.* 1992; Smith 1999; Hamilton-Bruce *et al.* 2002). Therefore, recovery of *N. albascopularis* (Etheridge, 1902) from the Aptian deposits of the Wallumbilla Formation (Doncaster Member) at White Cliffs gives this taxon the distinction of being both Australia's oldest definitively assigned non-marine gastropod and the earliest recorded representative of the Viviparidae in Australia. Recognition of *N. albascopularis* as a viviparid also serves to extend the family's temporal range in this region back to at least the later stages of the Early Cretaceous and, given its Jurassic–Recent fossil record in Europe, suggests a possible pre-Jurassic Pangean origin for the group. By the Early Cretaceous, the family had clearly diversified within the Gondwanan region, establishing key endemic taxa that still exist as descendant lineages today. This may be seen, to some extent, in the strong morphological similarity between *N. albascopularis* and other currently extant Australian

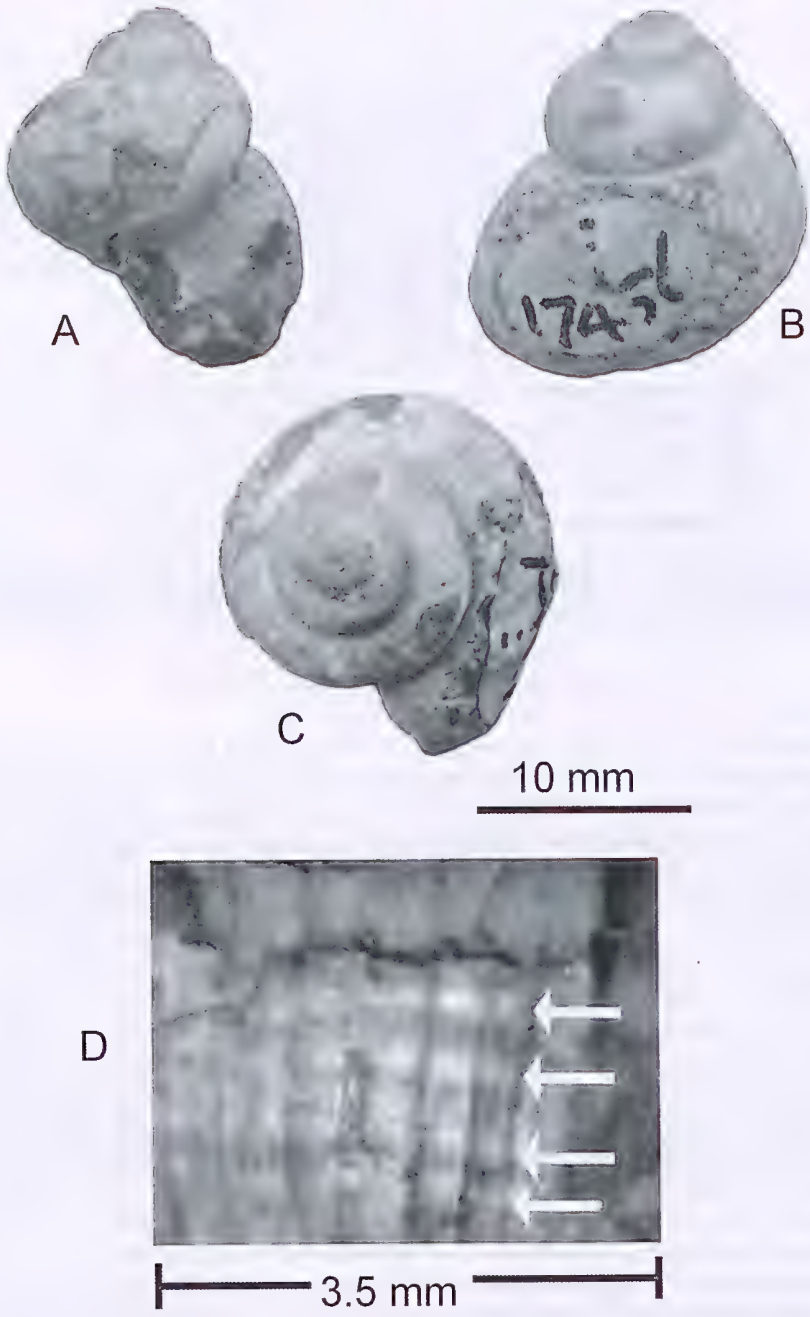

Fig. 2. AM 17456 *Notopala albascopularis* (Etheridge, 1902) in (*A*) apertural, (*B*) dorsal and (*C*) apical views. (*D*) Magnified section of last body whorl showing lirae (arrowed).

species of *Notopala*, particularly *N. hanleyi* and *N. sublineata* (Conrad, 1850) (see Fig. 1), both of which could potentially represent morphological derivatives. However, further study and the discovery of additional fossil material are required before any definitive phylogenetic relationship can be demonstrated.

Although there are numerous records of Cretaceous freshwater bivalves from Australia (McMichael 1957; Ludbrook 1985; Jell and Duncan 1986; Dettman *et al.* 1992; Hocknull 1997), there are very few for non-marine gastropods of the same period. Indeed, most of the better documented terrestrial units, including the Wonthaggi Formation, Eumeralla Formation and Koonwarra Beds (Korumburra Group) of Victoria (see Jell and Duncan 1986; Rich *et al.* 1988; Rich and Rich 1989) and Winton Formation of Queensland and SA (see Ludbrook 1985; Dettman *et al.* 1992; Hocknull 1997), have yet to produce any identifiable gastropod remains. The reasons for this apparent absence are unknown, but could be related to preservational biases, such as shells rapidly breaking up or dissolving after death. However, isolated specimens, such as the holotype of *N. albascopularis*, seem to have, on occasion, survived transport over considerable distances (perhaps on floating vegetation) prior to eventual burial. This scenario also appears to have been common for many of the other fossils at White Cliffs, which include a high proportion of terrestrial plant remains (Anderson 1892; Jaquet 1893; Etheridge 1902; Newton 1914), freshwater invertebrates (Etheridge 1902; Newton 1914; McMichael 1957; Dettman *et al.* 1992) and occasional freshwater/terrestrial vertebrates (White 1926; Molnar 1980, 1991; Kemp 1991), all probably derived from fluviatile input into the near-shore marine depositional environment.

Another factor possibly influencing the distribution of Early Cretaceous freshwater gastropods in Australia may have been the strongly seasonal cool to cold climates, which characterised many of the high-latitude continental (Douglas and Williams 1982; Gregory *et al.* 1989; Dettman *et al.* 1992; Cantrill 1998) and marine (Frakes and Francis 1988, 1990; Sheard 1990; Frakes *et al.* 1995; De Lurio and Frakes 1999) environments of the time. Although this may have limited the number of available habitats for non-marine gastropod species, it does not appear to have restricted overall taxonomic diversity or specimen numbers in the few deposits where they occur. Indeed, it is interesting to note that in the Lower Cretaceous freshwater river and lake deposits of Lightning Ridge, viviparids are one of the most common invertebrate faunal elements, far outnumbering other sympatric groups, such as thiarids, ellobiids and naticids (Smith 1999). The reasons for this apparent success are unknown, but could be related to the ability of the viviparids to give birth to live young and, thus, secure a competitive advantage over their contemporaries (however, larval brooding is also present in thiarids). Similarly, strict adaptation to freshwater may have enabled Cretaceous viviparids to rapidly colonise available upstream habitats. This contrasts with naticids and thiarids, whose Cretaceous record is largely derived from near-shore marine strata (see Etheridge 1902, Ludbrook 1966; Dettman *et al.* 1992; Stilwell 1998; Henderson *et al.* 2000), and may reflect a preference for more brackish water conditions around estuaries and coastal lagoons.

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Relationships of *Placostylus* from Lord Howe Island: an investigation using the mitochondrial cytochrome *c* oxidase 1 gene

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Abstract

Large (5–9 cm in length) land snails of the genus *Placostylus* are found in New Caledonia and the Loyalty Islands, northern New Zealand, the Three Kings Islands just north of New Zealand and on Lord Howe Island. Their presence on Lord Howe, an oceanic island less than 6 million years old, has been an intriguing biogeographical question. Maximum parsimony and maximum likelihood analyses using cytochrome *c* oxidase subunit I sequence data suggest that the Lord Howe Island and mainland New Zealand taxa are sisters, but that the Three Kings taxon is independently derived, possibly from New Caledonian stock. *Placostylus* colonies throughout the area of the present study are under considerable threat, with many intraspecific forms and some species threatened and some listed as endangered species. The taxonomic and conservation status of the Lord Howe Island populations are discussed.

Additional keywords: biogeography, New Caledonia, New Zealand, south-west Pacific, systematics.

Introduction

Oceanic islands typically have high levels of endemism and the origin of their species continues to be an intriguing question for evolutionary biologists. Notable examples among vertebrates of such studies include Darwin's finches of the Galapagos Islands, the honeyeaters of Hawaii (Freed *et al.* 1987) and *Brachylophis* on Pacific Islands (Gibbons 1981, 1985). There are also spectacular examples of radiations among invertebrates, some of the better known being the Pacific Island land snails, where there have also been massive human-induced extinctions (e.g. Solem 1990; Cowie 1992, 1996).

The likelihood of dispersal of individuals and taxa to oceanic islands is dependent on many factors, including the distance from source populations and the size and habitat complexity of the island. However, overriding all these factors is the dispersal ability of the taxon. Dispersal ability can be determined by intrinsic abilities (power of flight, body size and physiology (e.g. resistance to desiccation, tolerance of salt water, habits or habitat preferences; an arboreal species may be more likely to be transported by wind storms than a ground-living or burrowing species)) or extrinsic factors (prevailing winds, presence of suitable dispersal agents etc.). Land snails have no intrinsic means of long-distance dispersal, although small-sized species, in particular, may be dispersed aerially during major storms, accidentally carried by birds (Rees 1965; Vagvolgyi 1975; Kirchner *et al.* 1997) or rafted on floating vegetation. Successful long distance passive dispersal for most taxa is rare, especially once communities are established (Ward and Thornton 2000). The improbability of oversea dispersal for some taxa has led to hypotheses involving sunken continents or land bridges.

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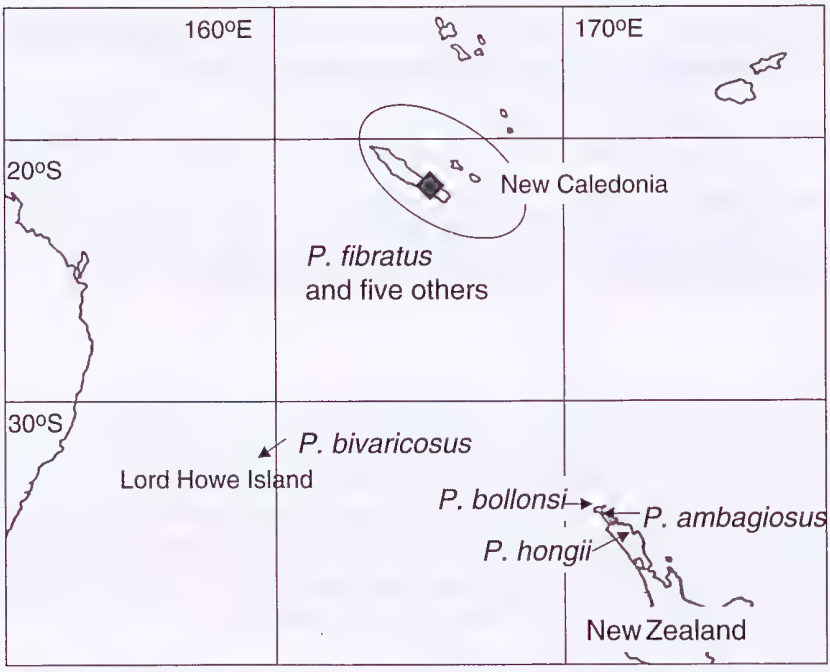

Fig. 1. The SW Pacific, showing the locations of the species of *Placostylus* included in the present analysis. The ellipse around New Caledonia is intended to show the overall range of the six species of *Placostylus* recognised from that area. The diamond indicates the locality of the New Caledonian specimen of *P. fibratus* used in the analysis.

Lord Howe Island (31°33′S, 159°05′E) lies in the Tasman Sea, is approximately 11 km long and rises to 875 m. It is nearest to eastern Australia, lying 700 km NE of Sydney and 496 km E of Port Macquarie, the nearest point on the coast of New South Wales (NSW). Norfolk Island is 890 km away and Auckland, New Zealand, is 1560 km away (Fig. 1). The highly endemic fauna and flora have elicited much discussion (see Paramonov 1958, 1960, 1963). The island is of volcanic origin, being formed between 6.4 and 6.9 million years ago (McDougall *et al.* 1981), and lies on the eastern edge of the Lord Howe Rise, a submarine fragment of eastern Australia that separated in the Cretaceous (Cook and Belbin 1978). Drill cores show that the Rise itself has never been above water (Van der Lingen 1973). A chain of submarine seamounts runs due north of Lord Howe Island. These are assumed to be progressively older northwards (McDougall *et al.* 1981).

Among the highly endemic fauna and flora is the extinct horned tortoise (*Meiolania platyceps* Owen, 1886) that was apparently terrestrial with poor swimming abilities (Gaffney 1983, 1996) and with close relationships to taxa in eastern Australia and New Caledonia (Walpole Island; Gaffney 1996). Notable among the endemic flora are three endemic genera of palms. There are many indigenous invertebrates, including over 80 species of land snails (Iredale 1944). Prominent among the land snails is *Placostylus bivaricosus* (Gaskoin, 1855), which reaches up to approximately 8 cm in length. Hedley (1892) stated that '… *Placostylus* appears a more fruitful subject of study [for biogeography] than any other molluscan genus inhabiting the same area' and the mystery of the presence of *Placostylus* on Lord Howe was also discussed by Etheridge (1891).

The distribution of *Placostylus* was such a conundrum to Hedley (1892) that he hypothesised that the islands containing these snails were '... portions of a shattered continent [which he called the Melanesian Plateau] and are connected by shallow banks formerly dry land'. This hypothesis met strong opposition from at least one notable contemporary, Alfred R. Wallace (see text of letter to Hedley; Wallace 1974).

In the most recent taxonomic review of the group (Haas 1935), species attributed to the genus *Placostylus* are found through some of the western Pacific Islands (New Zealand, New Caledonia, Solomon Islands, Fiji and Vanuatu). Haas (1935), who divided the genus into several subgenera, restricted the typical subgenus to New Caledonia (type species *Limax fibratus* Martyn, 1784; ICZN Opinion 1662 (ICZN 1992)). Several 'subgenera' are recognised that encompass the Pacific Island and New Zealand taxa, but the New Zealand–Lord Howe and New Caledonian (plus Loyalty Islands) taxa, with their large, solid shells, are more similar to one another than to the taxa further north, as noted by Hedley (1892). Because no other similar species are included in this grouping, it is reasonable to assume that the sister-taxon of the Lord Howe species is from either New Zealand, the Three Kings Islands or New Caledonia.

The New Caledonian (including Loyalty Islands) *Placostylus* are still relatively poorly known, both taxonomically and biologically. There are many names available in the literature, but no modern revisions have been published. Franc (1956) recognised 19 species but, using anatomical characteristics, Chérel-Mora (1982, 1983) reduced these to four, although most of her work remains unpublished. Dr E. Neubert, who is currently investigating the taxonomy and anatomy of the group in New Caledonia and the region, stated that, of approximately 140 available names, six valid species and approximately 20 geographic subspecies can be recognised in New Caledonia (Neubert 2001). The shell morphology of some of the New Caledonian taxa is very similar to that of Lord Howe Island and New Zealand taxa. The taxon included in the present analysis is *P. fibratus* (Fig. 2*E*,*F*), the type species of *Placostylus*. Many of the New Caledonian taxa are threatened (Neubert 2001) and some are still used for food. Salas *et al.* (1997) provide some data on the biology of *P. fibratus*.

While the systematics of the Lord Howe species is poorly understood (see Appendix), a recent survey of extant populations has been performed (Ponder and Chapman 1999), as well as a preliminary study of the genetics (Colgan and Ponder 2001).

Species similar to Lord Howe Island *Placostylus* occur in northern New Zealand (Powell 1947, 1951*b*; Choat and Schiel 1980; Triggs and Sherley 1993; Sherley 1996) and the Three Kings Islands (off northern-most New Zealand; Powell 1951*a*; Brook and Laurenson 1992). Members of the subgenus *Maoricolpus* Haas, 1935 (type species *Bulimus shongii* (= *hongii*) Lesson, 1830) include the species found in northern New Zealand and Lord Howe Island. The Three Kings Islands species (*P. bollonsi* Suter, 1908) is placed in a monotypic subgenus, namely *Basileostylus* Haas, 1935.

In New Zealand, there are three main taxa. *Placostylus hongii* (Fig. 2*A*) has been recorded from sites between Whangaroa and Whangarei on the mainland Northland and on offshore islands between Whangaroa and Great Barrier Island (Powell 1979; Browne 1980; Brook and McArdle 1999). *Placostylus ambagiosus* Suter, 1906 (Fig. 2*B*,*C*), comprising ten named extant subspecies (Powell 1947, 1951*b*), is located in the northern-most tip of the Northland Peninsula, where it is confined to tiny remnant populations. The third taxon, *P. bollonsi* (Fig. 2*D*), is confined to the Three Kings Islands that lie 60 km NW of Cape Reinga. This group is comprised of one large island and three small islands, all of which have colonies of this snail (Brook and Laurenson 1992). The biota of these islands also

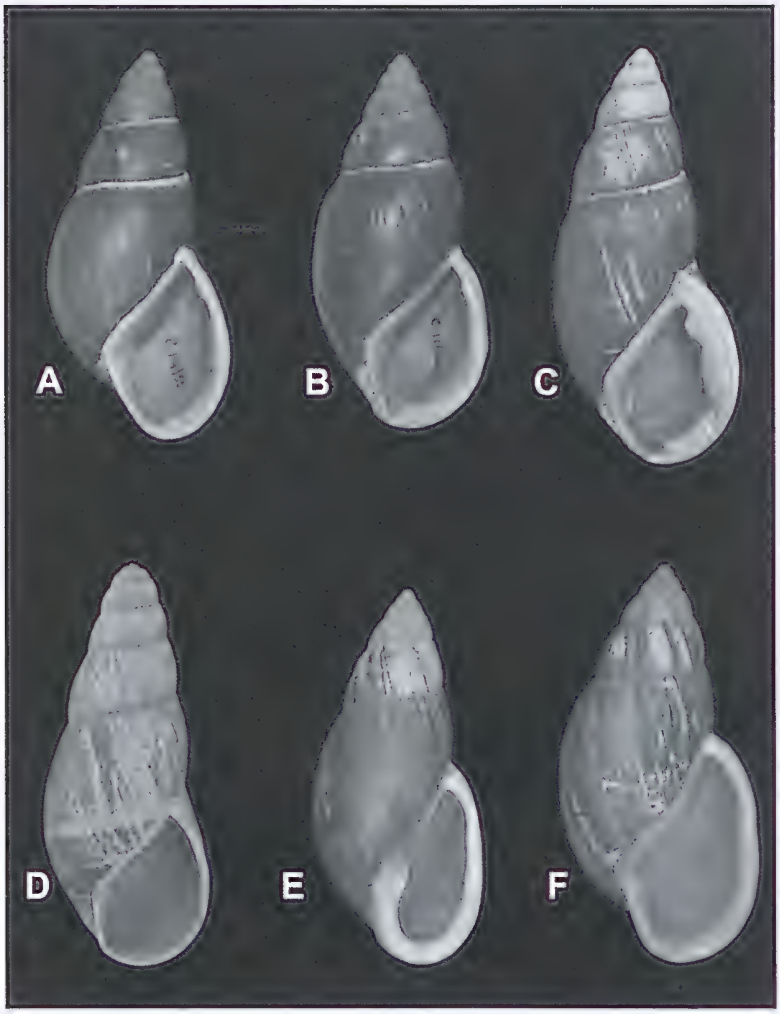

Fig. 2. Shells of *Placostylus* species (all material from the Australian Museum, Sydney, NSW; dimensions are maximum length). Images not to same scale. *A*, *P. hongii* (Lesson), Tauranga-Kawai Point, 6 km N of Whanaki, Northland, New Zealand, C.114955, 77.6 mm. *B*, *P. ambagiosus paraspiritus* Powell, paratypes, headland 1 mile S of Cape Maria van Diemen, Northland, C.115002, 64.4 mm. *C*, *P. ambagiosus whareana* Powell, paratype, valley to north of Whareana Stream, between Waikuku Beach and Parengarenga, Northland, C.115001, 82 mm. *D*, *P. bollonsi bollonsi* (Suter), paratype, Big King Island, Three Kings Islands, C.29117, 91.5 mm. *E,F*, *P. fibratus* (Martyn), Isle of Pines, New Caledonia, C.409415, 84 mm (*E*) and Bourail, New Caledonia, C.409409, 84 mm (*F*).

includes many other endemics. All the New Zealand species of *Placostylus* have received considerable taxonomic (Powell 1938, 1947, 1948, 1951*a*, 1951*b*; Climo 1973; Sherley 1996) and, more recently, conservation attention (Brook and Laurenson 1992; Triggs and Sherley 1993; Sherley 1996; Sherley *et al.* 1998), including the publication of a Recovery Plan (Parrish *et al.* 1995).

Some of the New Zealand taxa are critically endangered: the Three Kings species, *P. bollonsi*, is confined to two small populations distinguished as separate subspecies

(Powell 1948, 1951*a*; Climo 1973), one of approximately 130 individuals in a small area of only approximately 1.69 ha and the other of approximately 360 individuals in approximately 2.7 ha (Brook and Laurenson 1992). These populations are restricted by lack of suitable habitat and are not threatened significantly by predators. *Placostylus ambagiosus*, a species found only in far northern New Zealand, consists of a number of named subspecies (Powell 1938, 1947, 1951*b*) confined to tiny remnant populations that show some degree of genetic structuring using isozyme electrophoresis (Triggs and Sherley 1993). Some of these populations have fewer than 10 living individuals (Parrish *et al.* 1995) and all are threatened by introduced predators (rats, birds and pigs). *Placostylus hongii*, which lives south of *P. ambagiosus* in Northland, is known from a few mainland locations and some offshore islands and is extant from five mainland populations and three offshore island groups (Brook and McArdle 1999). Translocation of the two mainland species has been undertaken for conservation purposes (Sherley 1994).

The broader phylogenetic relationships of *Placostylus* within the Bulimulidae are still not fully understood. Solem (1959) argued for a relationship between *Bothriembryon*, the New Hebridean (i.e. Vanuatu) *Diplomorpha* and *Placostylus* based on anatomical data, with the latter genus having a northern origin. However, Breure (1979) placed *Placostylus* in a separate subfamily that he regarded as the sister-taxon to the rest of the family. He hypothesised that members of the Placostylinae reached New Zealand via east Antarctica and moved north. According to Breure (1979), placostylines show little relationship with the buliminids found in the western and southern parts of Australia (*Bothrembrion*; subfamily Orthalicinae).

The present paper provides evidence from molecular data relating to the question of the origin of the Lord Howe Island *Placostylus*. Because the 'subspecific' taxa (see Appendix) on Lord Howe Island are extinct, their status cannot be assessed using this methodology.

Material and methods

Material

A list of the specimens sequenced is given in Table 1. The sequenced specimen of *P. fibratus* came from a population with intermediate characteristics between *P. fibratus fibratus* and *P. fibratus souvillei* Kobelt, 1891 (Dr E. Neubert, personal communication). --

DNA extraction and sequencing

Lord Howe Island and New Caledonia specimens were extracted and sequenced in Sydney, whereas New Zealand specimens were extracted and sequenced at Landcare Research, Auckland.

For New Zealand specimens, foot muscle tissue was homogenised in 500 µL extraction buffer (0.01 M EDTA, 0.05 M NaCl, 0.5 M Tris-HCl, pH 8.0, 2% sodium dodecyl sulfate). A 10 µL aliquot of 10 mg mL^{-1} proteinase K (Boehringer, Mannheim, Germany) was added and the homogenate incubated at 65°C for 1 h. Samples were extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1), followed by extraction with chloroform/isoamyl alcohol (24:1). The DNA was ethanol precipitated and the pellet rehydrated in 50 µL buffer (10 mM Tris Cl (pH 7.4), 1 mM EDTA (pH 8.0)) following RNAse treatment.

A 655 bp region of the cytochrome *c* oxidase 1 (*CO1*) gene was amplified by polymerase chain reaction (PCR) using primers LCO-1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO-2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) designed by Folmer *et al.* (1994). Amplifications were performed in a volume of 50 µL and consisted of 10 pmol of each primer, 10 mM Tris-Cl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl and 0.2 mM of each dNTP. The addition of 2 units Taq polymerase (Boehringer) followed an initial step of 2 min denaturation at 94°C. Cycling consisted of denaturation at 94°C for 1 min, annealing at 50°C (*CO1*) for 1 min and extension at 72°C for 1 min and 30 s, for 35 cycles. A final cycle included a 5 min extension at 72°C.

The PCR products were purified using the QIAquick™ PCR direct purification kit (Qiagen, Venlo, The Netherlands), according to the manufacturer's instructions. Direct sequencing of purified products by the

Table 1. List of specimens sequenced and their localities

Specimen	Museum no.	Locality	Tissues
P. bivaricosus		Lord Howe Island	F
P1	C387658	Forest near Airport, 31°32′29″ 159°4′29″	F
P2	C387660	Stephen's Reserve, 31°31′28″ 159°3′56″	F
P3, 6	C387650	Lagoon foreshore, 31°31′41″ 159°3′47″	F
P4	C387742	Ned's Beach, 31°31′20″ 159°3′52″	F
P5	C387675	Hiscox property, 31°31′17″ 159°4′04″	F
P7–8	C387687	North Bay, 31°31′08″ 159°2′31″	F
P. ambagiosus		Far north, Northland, New Zealand	F
P. a. 'tirikawa' WG850-853	M02 877 512	Near Tirikawa Pa, 34°26′29′172°44′44′	DG, M
P. a. watti Powell GS 6	N01 148 543	North Cape, east, 34°24′47″ 173°02′47″	DG, M, Ov
P. a. annectens Powell			
GS 16, 17, 19	N02 048 527	Te Huka, 34°25′10″ 172°55′54″	DG, M
GS18, 20	N02 025 534	Matirarau, 34°25′17″ 172°54′24″	DG, M, Ov
P. a. lesleyae Powell GS 21-25	N02 901 483	Te Paki Trig, 34°28′03″ 172°46′18″	DG, M
P. hongii		Northland, New Zealand	
WG 862-865		Whangaruru North Head Scenic Reserve (35°22′20″ 174°21′45″), Northland, New Zealand	DG,M
P. bollonsi		Three Kings Islands, New Zealand	DG,M
P. b. bollonsi GS 26-30	L01 323 832	Three Kings, 34°09′06″ 172°08′40″	DG, M, Ov
P. b. caperatus Powell GS 31-35	L01 319 830	Three Kings, 34°09′13″ 172°08′25″	DG, M, Ov
P. b. arbutus Powell GS 36-40	L01 309 827	Three Kings, 34°09′22″ 172°07′46″	DG, M, Ov
P. b. 'northeast' GS 41-45	L01 343 854	Three Kings, 34°07′55″ 172°09′59″	DG, M, Ov
P. fibratus		New Caledonia	
	C409417	On the SW slope of Monts Koghis at approximately 500 m, 22°10′S 166°32′E	F

DG, Digestive gland; F, foot, M, mantle; Ov, ovary.
The New Zealand material is lodged in the New Zealand Arthropod Collection at Mt Albert, Auckland; other voucher material is housed in the Australian Museum, Sydney, NSW.

primers used for the original amplification was achieved using the Prism Ready Reaction Dye Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Norwalk, CT, USA), following the manufacturer's instructions. Sequences were analysed on an automated DNA sequencer (model 377; Applied Biosystems). Two independent PCR products from each specimen were sequenced in both directions.

For the Sydney experiments, molecular biological procedures generally followed the methods of Colgan *et al.* (2000*a*, 2000*b*). The DNA extraction of foot muscle followed the hexadecyltrimethylammonium bromide procedure of Saghai-Maroof *et al.* (1984).

The Sydney primer pair for *CO1* is listed below with degeneracies written with standard IUB codes. Numbers in parentheses indicate the 5′ end of the sequence in the complete mitochondrial genome of the earthworm *Lumbricus terrestris* (Boore and Brown 1995): COX1AF, (19) CWAATCAYA AAGATATTGGAAC; COX IAR, (726) AATATAWACTTCWGGGTGACC. Polymerase chain reaction cycling used the same basic protocols as Colgan *et al.* (2000*a*, 2000*b*), with a general reaction mix of 1.0 U Red Hot™ thermostable DNA polymerase, buffer IV (10×, 20 mM $(NH_4)_2SO_4$, 750 mM Tris-HCL, pH 9.0, 0.1% (w/v) Tween; Advanced Biotechnologies, Leatherhead, Surrey, UK), 0.05 mM dNTPs, 2.5 mM $MgCl_2$,12.5 pmol of each primer and 1 μL of a dilution (usually 1:10) DNA sample in a reaction volume of 50 μL overlaid with 30 μL oil. The usual cycling profile was denaturation at 95°C for 4 min, annealing at 45°C for 45 s and extension at 72°C for 30 s (one cycle), followed by 32 cycles of 95°C for 30 s, 45°C for 45 s and 72°C for 30 s and one cycle of 95°C for 30 s, 45°C for 45 s and 72°C for 5 min. The PCR products were purified using the QIAquick™ PCR Purification Kit and sequenced in both directions with an automated DNA sequencer (ABI® 310; Applied Biosystems) using the BigDye™ version 2.0 sequencing kit.

Alignment and sequence composition

Sequences were edited using Sequence Navigator version 1.0.1 (Anonymous 1994). A compilation of all sequences was aligned using the default values for parameters in CLUSTAL W (Thompson *et al.* 1994). It was not necessary to alter this alignment manually. Data were compiled using MacClade (Maddison and Maddison 1992).

Maximum parsimony was conducted in PAUP* 4.0b9 (Swofford 2000) with the default conditions for parsimony analyses with branch and bound searches guaranteed to find the shortest trees. All characteristics were unordered and unweighted. The steepest descent option was not enforced and accelerated transformation for character optimisation was assumed. Zero length branches were collapsed to give polytomies. Gaps were treated as unknown in all analyses. Bootstrap pseudo-sampling was conducted for 100 replicates with branch and bound searching in each. Shortest trees satisfying alternative hypotheses of relationships were sought with the same strategy but using constraints in PAUP.

For maximum likelihood analyses in PAUP*, a Hasegawa–Kishino–Yano (HKY) invariant model was used with the following settings. The number of substitution types was two, with the transition transversion ratio estimated by maximum likelihood. Empirical nucleotide frequencies were used. The proportion of invariable sites was estimated via likelihood and the distribution of rates at variable sites was assumed equal. The molecular clock model was not enforced. Other parameters took the default values in PAUP* 4. Comparison of trees using Kishino–Hasegawa likelihood tests assumed a normal approximation, with a two-tailed test. Shimodaira–Hasegawa tests used a one-tailed resampling of estimated log-likelihoods bootstrap with 1000 replicates.

Outgroup sequences were obtained from the helicoidean pulmonates *Aegista scepasma* (Bradybaenidae) (Genbank Accession AB024900; Shimizu and Ueshima 2000), *Cepaea nemoralis* (Helicidae) (Genbank Accession U23045; Yamazaki *et al.* 1997), *Euhadra herklotsi* (Bradybaenidae) (Genbank Accession Z71701; Yamazaki *et al.* 1997) and *Albinaria caerulea* (Genbank Accession NC_001761; Hatzoglou *et al.* 1995).

Morphometrics

Shell measurements were made using callipers. The discriminant analysis was undertaken using SYSTAT™ version 10 (SPSS, Chicago, IL, USA).

Results

The locations of samples are shown in Table 1.

GenBank accession numbers for the *CO1* sequences used are AY165836–AY165843, AY165852 and AY290737–AY290745. The alignment used in the analysis is available from

the authors. As usual with mitochondrial sequences, there is a major AT bias in nucleotide composition. The mean overall percentages of nucleotides is as follows: A, 26.844; C, 14.653; G, 17.174; T, 41.328 (overall AT average 68.17%).

The probability that the nucleotide composition of the sequences is homogeneous is very close to 1 ($\chi^2 = 37.600$, d.f. = 63, $P = 0.99$), suggesting that variation in base composition between taxa has little effect on the analyses.

The levels of pairwise difference of the HKY measure of genetic distance within the *Placostylus* samples vary between 0.0188 (within Lord Howe Island) and 0.6278 (between *P. ambagiosus annectens* and *P bollonsi* 'north-east') within New Zealand samples. In Table 2, the averages of the Kimura two-parameter distance are given for comparisons within and between various species. Within species, the averages are less than 0.1, but distances between species rise to 0.2014 for the comparison of Lord Howe Island snails with *P. bollonsi.*

The results are illustrated in Figs 3, 4. The consensus of the maximum parsimony trees (Fig. 3) includes the following strongly supported monophyletic clades: Lord Howe Island specimens (with a decay index of 27), *P. ambagiosus*, *P. ambagiosus* + *P. hongii* and *P bollonsi.* Notably, the New Zealand samples are split into two distinct lineages. Constraining these to be monophyletic requires five more steps than the unconstrained, most parsimonious trees. The Lord Howe Island samples are sister to the *P. ambagiosus* + *P. hongii* clade. This group is, in turn, sister to the New Caledonian sample. Four extra steps are required to make the Lord Howe specimens sister to this to the exclusion of the New Zealand snails. A sister pairing of Lord Howe and New Caledonia specimens is seen in only 6.6% of bootstrap trees compared with 38% of bootstrap replicates showing the pairing of Lord Howe Island with *P. ambagiosus* + *P. hongii.* The sister pairing of New Zealand *P. ambagiosus* + *P. hongii* with New Caledonia or all the New Zealand taxa with New Caledonia requires, respectively, two and five more steps than the maximum parsimony tree. Comparisons of the likelihoods of the trees satisfying various constraints are shown in Table 3 and the maximum likelihood tree is shown in Fig. 4. In particular, the Kishino–Hasegawa tests reject the possibility that there is a single New Zealand radiation that is

Table 2. Average of pairwise genetic distances within and between *Placostylus* species using the Kimura two-parameter genetic distance

Comparisons	Average
Within species	
P. ambagiosus	0.0144
P. bivaricosus	0.0630
P. bollonsi	0.0028
Between species	
P. ambagiosus/*P. bivaricosus*	0.1581
P. ambagiosus/*P. bollonsi*	0.1755
P. ambagiosus/*P. fibratus*	0.1345
P. ambagiosus/*P. hongii*	0.0572
P. bivaricosus/*P. bollonsi*	0.2014
P. bivaricosus/*P. fibratus*	0.1717
P. bivaricosus/*P. hongii*	0.1494
P. bollonsi/*P. fibratus*	0.1849
P. bollonsi/*P. hongii*	0.1732
P. fibratus/*P. hongii*	0.1325

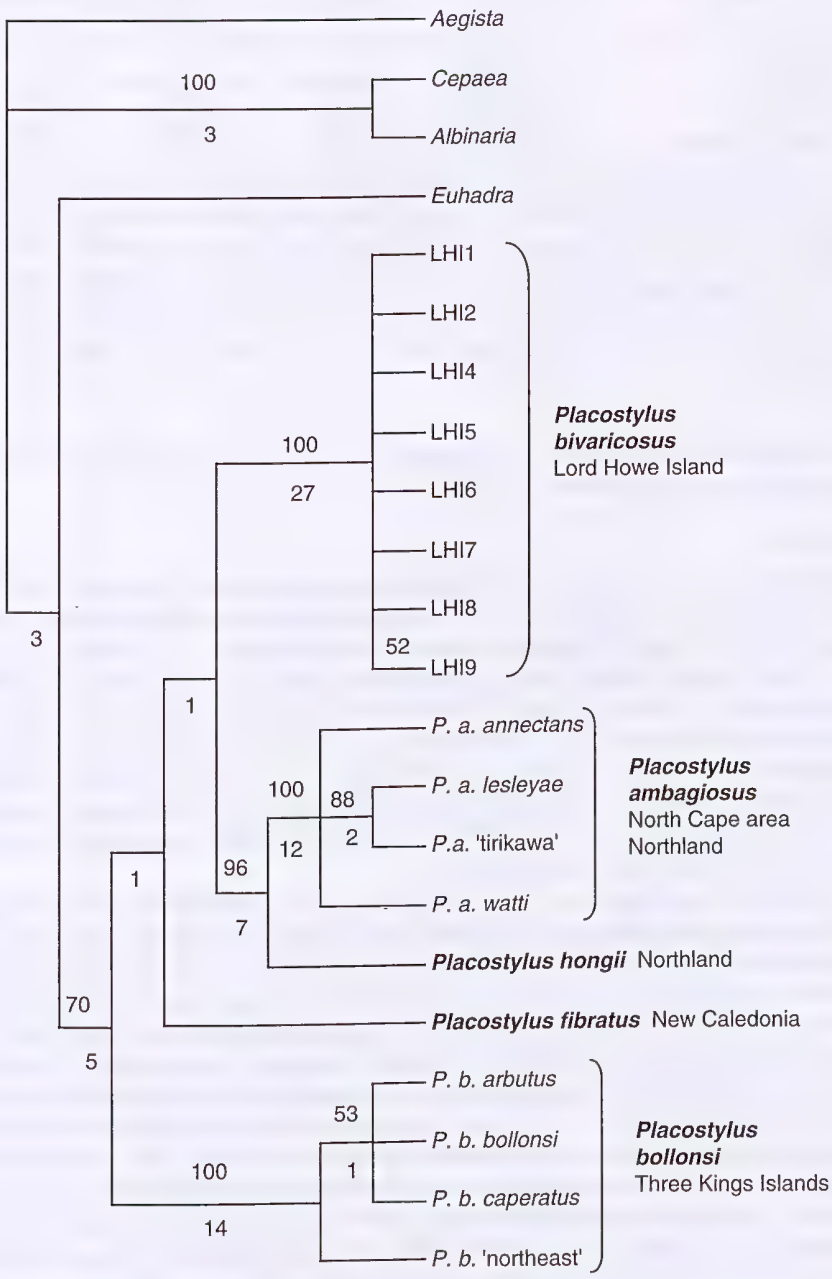

Fig. 3. The strict consensus of 50 trees found in a branch and bound search of the part cytochrome *c* oxidase 1 gene. The trees were 556 steps long with a consistency index of 0.678. Bootstrap percentages above 50% are written above branches and Bremer decay indices below. The species names for *Placostylus* are indicated in bold.

Table 3. Comparisons of the likelihoods of trees constrained to show various relationships

Tree	–ln L	Diff –ln L	Kishino–Hasegawa	Shimodaira–Hasegawa
Unconstrained	3019.61928			
NC with NZ	3025.29525	5.67597	0.000*	0.182
NC with LHI	3023.03754	3.41826	0.000*	0.346
LHI with NC + part NZ	3023.02717	3.40789	0.000*	0.339

The first column shows the imposed constraint (if any), the second column shows the negative of the log likelihood of the maximum likelihood (ML) trees, the third column shows the difference between tree likelihoods and the last two columns give the probabilities that the compared trees have the same likelihood under the Kishino–Hasegawa (two-tailed) test and the Shimodaira–Hasegawa (one-tailed) test. The constraints are that: (*1*) New Caledonia (NC) and all New Zealand (NZ) specimens are a sister pair; (*2*) New Caledonia and all Lord Howe Island (LHI) snails form a sister pair; and (*3*) the Lord Howe Island snails are sister to a group comprising the New Caledonian sample and New Zealand *P. hongii* and *P. ambagiosus*.

sister to the New Caledonian sample (constraint 1) or that this latter specimen and the Lord Howe Island snails are sister taxa (constraint 2).

Discussion

New Caledonia and New Zealand were separated by the end of the Cretaceous (Hall 2002), but it is unclear as to how much of the Norfolk Ridge remained emergent through the early Tertiary. The oldest basalts on Lord Howe Island were formed only 6.4 million years ago. Thus, the geological evidence indicates that dispersal, rather than vicariance, must account for the presence of *Placostylus* on Lord Howe Island. While we can only speculate about possible dispersal mechanisms, an analysis such as this can provide evidence for the likely origin of the source for the dispersal event. This does not eliminate the probability that past intermediate populations, for example, on islands that are now submerged seamounts, ridges or plateaus to the north of Lord Howe Island (or the Three Kings Islands), may have facilitated dispersal as 'stepping stones'.

The Three Kings Islands are comprised of indurated, deformed marine sedimentary and volcanic rocks of probable early Cretaceous age (Haywood and Moore 1987; Brook and Laurenson 1992). Brook and Laurenson (1992) state that '... it is possible that marine conditions persisted in the vicinity of present day Three Kings Islands for much or all of that time' (Cretaceous to early Caenozoic). The present configuration of northern New Zealand (including the Three Kings) was probably developed during the mid-Caenozoic, with a subaerial peninsula developed from about Kaitaia/Doubtless Bay to the Three Kings area in the early Miocene (15–20 million years ago), this degenerating to a few islands by the mid-late Miocene (Brook and Thrasher 1991). The northern-most end of Northland ('North Cape') was, at that time, a separate island and was connected to the rest of Northland subsequent to the early Pliocene (5–3 million years ago; Pillans *et al.* 1992).

Whereas dispersal must be invoked to account for the presence of *Placostylus* on Lord Howe Island, the Three Kings Islands could, seemingly, have been connected or nearly connected to Northland in the mid-Tertiary and closely adjacent (approximately 10 km) during interglacial periods in the Pleistocene (Brook and Laurenson 1992). Given these data, postulating an origin of *P. bollonsi* from Northland seems logical and has been argued for by Brook and Laurenson (1992). However, there is little support for this hypothesis from our data, which suggest that the Three Kings taxon and the mainland taxa represent two

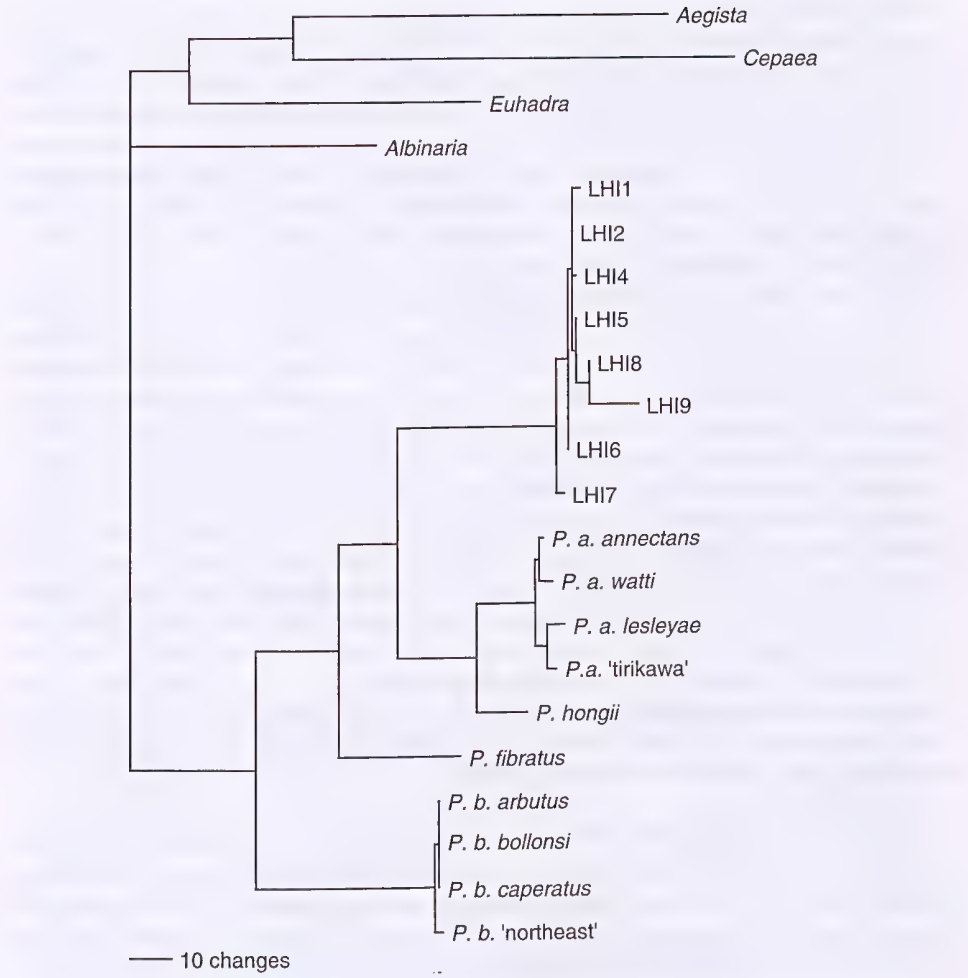

Fig. 4. Phylogram for the cytochrome *c* oxidase 1 data of the maximum likelihood tree resulting from an heuristic search with 10 replicates of random sequence additions. See Fig. 3 for details of taxa.

separate clades, as reflected in the allocation of different subgeneric names. It appears that the Three Kings taxon is the result of either a long overseas dispersal event or (more probably, in our opinion) it represents a surviving relict of a taxon distributed on now submerged islands along the Norfolk Ridge.

Our results clearly suggest a sister relationship between the northland *P. ambagiosus* and *P. hongii*, separated by the late Pliocene isthmus between what was once a northern island. Whereas there is strong support for the monophyly of each of the main radiations (*P. ambagiosus*, *P. hongii*; *P. bollonsi* and *P. bivaricosus*), regrettably, the analysis is weakened by there being only a single sample from New Caledonia.

Brook and McArdle (1999) have advocated pre-European Maori transport of *Placostylus hongii* (which was used as a food item; Haywood and Brook 1981) to and from offshore islands to account for their present distribution. There is no evidence of pre-European contact with Lord Howe Island, so some other form of overseas dispersal must have occurred. This most likely occurred as a result of an individual being carried in a

severe storm to the island. Whereas this may have come from a nearby population on an island (that has since disappeared) to the north, given that there is a chain of seamounts northwards, the results of our analysis suggest that there was a common origin with the mainland New Zealand taxa. Self-fertilisation is common in pulmonate snails, so even a single juvenile could establish a population. *Placostylus* hatchlings are known to be often arboreal, this habit facilitating such a mode of dispersal of this otherwise ground-dwelling species. Extremely rare aerial dispersal of hatchlings and juveniles, less than 1 cm in length, during major storms seems, to us, to be a more feasible explanation of dispersal than other hypotheses invoking rafting or transport by birds.

The results show low levels of genetic divergence between individual Lord Howe Island sequences (Fig. 3) but, on the available small amount of material, no clear pattern emerges (*16S* rDNA sequences are also available for the Lord Howe Island samples and show substructure between the available populations (Colgan and Ponder 2001)). There is considerable variation in shell morphology within the area containing the material used in the analysis (Ponder and Chapman 1999) but, again, the material sampled is not adequate to address questions relating to whether there is any underlying genetic basis for this variation. The range of the available samples from Lord Howe Island does not cover all the island, with none coming from the hills and mountains in the southern part where *Placostylus* appears to be extinct. While it remains possible that more southerly populations, from the remote areas around Mounts Lidgbird and Gower, may still be extant, it remains untested whether they are genetically distinct, as suggested by their shell morphology, or whether there is a significant geographic barrier to gene flow in this region. Triggs and Sherley (1993) showed that there was a considerable level of genetic variation between populations of *P. ambagiosus*. In these cases, small but overlapping differences in shell morphology led to prior creation of several subspecies (Powell 1938, 1947, 1948, 1951*b*; Sherley 1996).

The genetic status of New Zealand *Placostylus* species has been assessed by allozyme electrophoresis (Triggs and Sherley 1993). Evidence of distinct lineages (possibly at the species level) was found in one mainland morphospecies, *P. ambagiosus*, over a geographic scale of tens of kilometres, with differentiation apparently fostered by Pleistocene and late Pliocene sea level changes. These authors found no significant variation in allozymes in *P. bollonsi* from the Three Kings Islands, but the results presented herein suggest that at least the north-east population may be distinct.

The results clearly discount the Lord Howe Island taxon being a recent introduction from New Zealand but, because we have only one specimen from New Caledonia, where there are probably six Recent species present, the possibility of a relatively recent introduction from that source cannot be discounted.

Conservation status of Lord Howe Island Placostylus

Etheridge (1889) noted that *Placostylus* on Lord Howe Island was '… found everywhere under cover, and in immense numbers' and that it '… appears to avoid open spaces as a rule, and prefers shady damp situations and the scrubby hill sides where composed of the Coral-sand rock. It is sparingly represented even at the higher altitudes, being reported as seen under the 'wall' of Mount Ledgbird.' Hedley (1891) noted that it was '… all over the island in sheltered places under stones; abundant'.

However, this previously abundant animal has been the subject of conservation concern for some time. Iredale (1944) noted that there was a '… new difficulty of recent years, the destruction effected by the rat plague, written up by Hindwood'. More recently, Smithers

et al. (1977) noted that *Placostylus* is apparently '... existing in only four small colonies, whereas from early reports it was dispersed widely from sea-level up to the mountain tops'. Ponder and Chapman (1999) reported that all the living specimens found in their 1999 survey were located on low, rather flat ground with ample litter. In most cases, the samples were taken on calcarenite soils or sandy soils derived, in large part, from calcarenite. Living specimens were found under well-developed litter. *Placostylus* seem to prefer the vicinity of the large native fig trees, but not exclusively so.

Placostylus bivaricosus is listed as endangered in NSW under the Threatened Species Conservation Act and a Recovery Plan (NSW National Parks and Wildlife Service 2000) has been prepared.

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Appendix. The Lord Howe Island *Placostylus* taxa

Placostylus bivaricosus (Gaskoin, 1855) is the only currently recognised species found on Lord Howe Island (Smith 1992). The last review of this group was by Iredale (1944), who noted that the: 'variation in this group is worthy of study … At the present time the various forms may be less visible in the field than in Roy Bell's time, when he indicated no less than six different colonies, separable in the field'.

No critical examination of the taxonomy of the Lord Howe Island *Placostylus* has ever been undertaken, although Smith (1992) catalogued the taxa and details of original names and references can be obtained from that source. Material in the Australian Museum collections appears to support the recognition of at least three, possibly four (see below), Recent and one fossil taxon on the basis of shell morphology (Ponder and Chapman 1999). These can tentatively be treated as 'subspecies' as follows.

Placostylus bivaricosus bivaricosus (Gaskoin, 1855)

Bulimus bivaricosus Gaskoin, 1855. Lord Howe Island (location of type material not known; fide Smith 1992: 108).

Placostylus bivaricosus belli Iredale, 1944. Lord Howe Island (holotype Australian Museum C.38973; Fig. 5*C*).

Remarks

The typical subspecies (Fig. 5*A*,*B*) is found on the northern end of the island and in the settlement area as far south as Intermediate Hill. *Placostylus bivaricosus belli* (Fig. 5*C*) is based on a small, narrow (shell length 54.6 mm, width 22.2 mm, aperture length 24.3 mm) specimen that can be matched with other occasional small specimens found within the settlement area.

Placostylus bivaricosus etheridgei (Hedley, 1891)

Bulimus bivaricosus etheridgei Hedley, 1891. Under the wall of Mt Lidgbird (Hedley, 1891). Iredale (1944) states that this species is 'apparently from the saddle of Mt Lidgbird' (three syntypes, Australian Museum C.33308; Fig. 5*H*).

Placostylus etheridgei 'Brazier', 1889. *Nomen nudum* (introduced as a name on the caption to a figure in Etheridge, 1889).

Placostylus bivaricosus royi Iredale, 1944. Little Slope, base of eastern side of Mount Gower (holotype, Australian Museum C.38974; Fig. 5*I*).

Remarks

This taxon is found in the southern mountains. It is variable in size, although often large, and has a thin shell with a weakly developed apertural varix and the surface of the shell is sculptured with oblique rugae. It is probably extinct, no living specimens having been collected for several decades. There is some suggestion from museum material of overlap, or perhaps merging, of typical *bivaricosus* and *etheridgei* in the area around Intermediate Hill. Unfortunately, the populations in this area are now either extremely reduced or, more likely, extinct. Both the available names for this taxon are from populations around the base of Mount Lidgbird and Mount Gower, or in the Erskine Valley, which lies between the two mountains. These have a significantly larger shell than samples from the top of Mount Gower (Fig. 5*D*,*F*; see below).

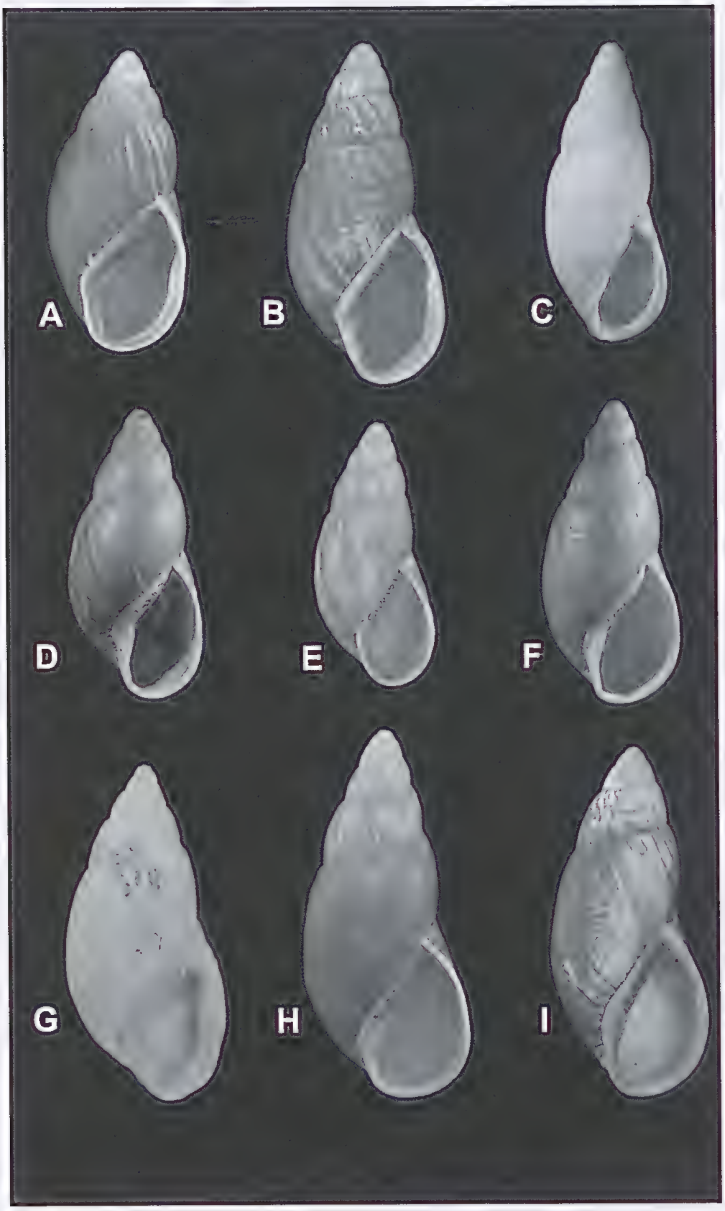

Fig. 5. Shells of *Placostylus* from Lord Howe Island (all material from the Australian Museum, Sydney, NSW; dimensions are maximum length). Images not to same scale. *A,B, P. bivaricosus bivaricosus* (Gaskoin), Lord Howe Island, C.114669, 56.6 mm (*A*) and C.114666, 60.7 mm (*B*). *C*, Holotype of *P. bivaricosus belli* Iredale, Lord Howe Island, C.38973, 54.6 mm. *D,F, P.* aff. *bivaricosus etheridgei* (Hedley), Mount Gower (C.114677), 54.0 mm (*D*) and 62.2 mm (*F*). *E, P. bivaricosus cuniculinsulae* (Cox), Rabbit (= Blackburn) Island, Lord Howe Island, C.118151, 45.6 mm. *G, P. bivaricosus solidus* (Etheridge), syntype, Lord Howe Island, C.171111, 74.8 mm. *H,I, P. bivaricosus etheridgei* (Hedley), syntype of *Bulinus etheridgei* Hedley, Mt Lidgbird, Lord Howe Island, C.33308, 67.2 mm (*H*) and holotype of *P. bivaricosus royi* Iredale, Little Slope, Lord Howe Island, C.38974, 77.2 mm (*I*).

Placostylus bivaricosus cuniculinsulae (Cox, 1872)

Bulimus (*Placostylus*) *cuniculinsulae* Cox, 1872. Rabbit Island (= Blackburn Island), Lord Howe Island (holotype, Natural History Museum, London, 1888.12.11.47–8).

Remarks

This small form was found only on Blackburn Island and is now extinct (Iredale 1944). Etheridge (1889) noted that there was a great deal of variation in shell morphology and that it seemed '... more in keeping with the facts to regard the Rabbit Island shell simply as a variety'. However, the material in the Australian Museum from Blackburn Island all possesses a distinctive, thin shell and is smaller than any adults seen from the main island (Figs 5*E*, 6; Table 4).

Placostylus bivaricosus solidus (Brazier in Etheridge, 1889)

Bulimus bivaricosus solida Brazier in Etheridge, 1889. (Seven syntypes, Australian Museum C.171111 (Fig. 5*G*), C.171110).

Remarks

This large, heavy form is found in calcarenite along the eastern side of the settlement area of the island. Smith (1992) indicates that the original introduction of the name is a *nomen nudum*. However, there is a statement differentiating the taxon in the original introduction of the name (p. 27), so it is valid. In addition, Etheridge (1891) described this taxon in greater detail and provided figures. There are also fossil specimens that appear to be indistinguishable from the Recent material (including one of the syntypes in C.171111), although specimens can usually be readily separated into the *solida* morph or the Recent morph. Unfortunately, there has not yet been any attempt to systematically collect fossil *Placostylus* and relate shell morphology to stratigraphy.

A comparison of three basic shell measurements (Table 4) indicates that the Recent taxa discriminate well (Fig. 6), but mainly on size rather than shape differences. There also appears to be justification for the recognition of an additional unnamed and recently extinct taxon that lived on the top of Mount Gower (Australian Museum C.114677; Fig. 5*D*,*F*) and on a razor-back spur to the south of the summit (Australian Museum C.114679). These two lots have similar shell dimensions to typical *P. bivaricosus* (Fig. 6; Table 4), but have the

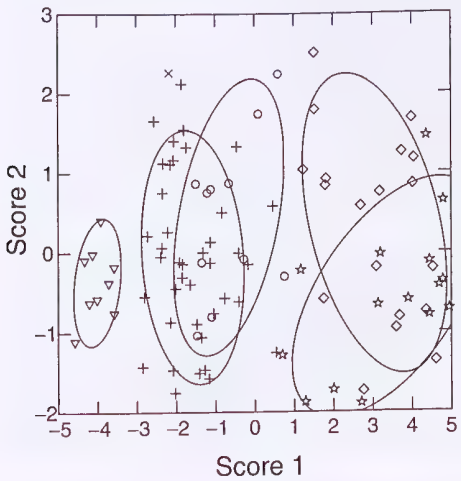

Fig. 6. Plot of the first and second discriminant scores from the measurement data summarised in Table 4. The ellipses have a confidence level of $P = 0.683$. (▽), *Placostylus bivaricosus cuniculinsulae*; (+), *P. bivaricosus bivaricosus*; (◇), *P. bivaricosus etheridgei*; (○), *P. bivaricosus* aff. *etheridgei* (top of Mt Gower); (☆), *P. bivaricosus solidus*.

Table 4. Shell measurements of *Placostylus* from Lord Howe Island
Data show the range and mean ± s.d.

	Shell length (mm)	Shell width (mm)	Aperture length (mm)
P. bivaricosus bivaricosus ($n = 40$)	46.0–61.9, 53.4 ± 3.5	21.3–27.5, 23.8 ± 1.5	23.9–32.4, 26.9 ± 2.1
P. bivaricosus etheridgei ($n = 19$)	65.5–77.1, 71.5 ± 4.1	28.0–35.3, 31.9 ± 2.3	31.8–41.1, 35.9 ± 2.9
P. bivaricosus cuniculinsulae ($n = 9$)	40.0–45.7, 43.3 ± 1.8	19.4–21.9, 20.3 ± 0.8	20.3–22.9, 21.4 ± 0.9
Summit of Mount Gower ($n = 11$)	51.9–65.1, 57.6 ± 4.2	23.7–27.8, 25.4 ± 1.2	26.5–31.9, 28.3 ± 1.8
P. bivaricosus solidus ($n = 15$)	61.1–79.0, 71.4 ± 6.2	26.8–36.6, 32.6 ± 3.1	31.5–41.0, 37.2 ± 2.7

Specimens measured: *P. b. bivaricosus* (C118724; C114653; C.78759; C.114656); *P. b. solidus* (C.171111; C.171110; C.114648; C.47513); *P. b. etheridgei* (C.33308; C.38973; C.114672; C.114675); *P. b. cuniculinsulae* (C.118151; C.114646; C.114647); Mount Gower (C.114679; C.114677).
Aperture length is the external, not internal, length.

colour and surface sculpture of *P. bivaricosus etheridgei*. Given their isolation on the summit, they were probably genetically distinct from the populations around the base of the southern mountains and the typical lowland form.

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Short contribution

Changes in tissue composition during larval development of the blacklip pearl oyster, *Pinctada margaritifera* (L.)

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Abstract

This paper reports on the changes in proximate composition (i.e. protein, lipid and carbohydrate) of tropical blacklip pearl oyster, *Pinctada margaritifera* (L., 1758), larvae throughout development. Protein was the largest component of dried larval tissues. Mean protein, lipid and carbohydrate contents all decreased from Day 1 to Day 4. Lipid loss between Day 1 and Day 4 contributed 56% of the total energy utilised during this period, whereas protein contributed almost 40%. Between Day 18 and Day 21, the accumulation of lipid contributed almost 70% of the total energy gain per larva during this period, suggesting that lipid may be the primary energy reserve utilised during metamorphosis. Patterns of energy reserve composition, utilisation and accumulation within *P. margaritifera* larvae were comparable to those reported for temperate species.

Introduction

Fundamental differences exist between tropical and temperate marine environments that directly influence the organisms living within them. Tropical marine environments are characterised by surface-water temperatures of 20–30°C, relatively low nutrient loads and correspondingly low phytoplankton levels (Nybakken 1982). In contrast, temperate marine environments are characterised by seasonal, but relatively high, nutrient loads, high phytoplankton levels and water temperatures fluctuating between 10 and 20°C in a seasonal fashion (Nybakken 1982). On this basis, the rates of energy-metabolism in temperate bivalve molluscs cannot be assumed to pertain to tropical species. However, studies reporting on changes in proximate composition during larval development of bivalves have focused on temperate species (Holland and Spencer 1973; Bayne *et al.* 1975; Gallager and Mann 1986; Gallager *et al.* 1986; Whyte *et al.* 1987, 1990, 1991). The hypothesis tested in this study was that patterns of energy-reserve utilisation and accumulation in larvae of the tropical blacklip pearl oyster, *Pinctada margaritifera* (L., 1758), differ from those reported for temperate species. This was examined by determining changes in proximate composition (i.e. protein, lipid and carbohydrate) during larval development.

Materials and methods

Spawning induction and larval rearing of *P. margaritifera* followed standard procedures (Southgate and Beer 1997). Ten hatchery-conditioned *P. margaritifera* broodstock were induced to spawn using thermal shock. Progeny from each of the females were randomly distributed across larval batches. Larvae were reared in six 500-L fibreglass tanks and water was changed every 48 h. Larvae were fed a diet consisting of the two prymnesiophytes, *Pavlova salina* and *Isochrysis* aff. *galbana* clone T-ISO, and the diatom *Chaetoceros muelleri* (Southgate and Beer 1997). A sample of approximately 15000 larvae was taken for

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proximate analysis at each water change from each tank and stored in liquid nitrogen to await analysis of protein, lipid and carbohydrate content (Mann and Gallager 1985; Baethgen and Alley 1989). Prior to analysis, larval samples were freeze-dried. The experiment ended after 21 days, when 50% of larvae were 'eyed'. Water temperature was measured at 0800 hours and 1800 hours each day and ranged from 25°C to 29°C with a mean of 27.2°C.

Proximate compositional data were used to calculate total energy values using caloric equivalents of 36.42, 23.86 and 17.16 kJ g^{-1} for lipid, protein and carbohydrate respectively (Brett and Groves 1979).

Results and discussion

Pinctada margaritifera larvae increased in mean (± SEM, $n = 30$) shell length from 76 ± 1 µm on Day 1 (24 h after fertilisation) to 213 ± 5 µm on Day 21. Mean (± SEM, $n = 30$) larval dry weight increased from 556.6 ± 1.1 ng $larva^{-1}$ on Day 1 to 2793.4 ± 40.0 ng $larva^{-1}$ on Day 21. Mean survival of larvae to Day 21 was 13.2 ± 5.0%.

Changes in mean protein, lipid and carbohydrate content of *P. margaritifera* larvae are shown in Fig. 1. Protein was the largest component of dried larval tissues. Mean protein content almost halved from 133.7 ± 5.1 ng $larva^{-1}$ on Day 1 to 67.3 ± 3.1 ng $larva^{-1}$ on Day 4 (± SEM, $n = 6$). The most notable increase in mean protein content of larval tissues occurred between Day 12 (133.5 ± 17.7 ng $larva^{-1}$) and Day 18 (395.2 ± 12.9 ng $larva^{-1}$). Subsequently, the increase in protein content slowed considerably; the tissues of 21-day-old larvae possessed 417.7 ± 16.78 ng $larva^{-1}$ of protein.

Lipid was the second largest component of dried larval tissue during development. Mean lipid content decreased by greater than 75% from 73.3 ± 9.1 ng $larva^{-1}$ to 11.7 ± 1.1 ng $larva^{-1}$ between Day 1 and Day 4. Subsequently, larval lipid content increased to 96.8 ± 11.6 ng $larva^{-1}$ on Day 15, declined to 76.9 ± 5.2 ng $larva^{-1}$ on Day 18, then increased rapidly to 140.6 ± 2.2 ng $larva^{-1}$ on Day 21.

Carbohydrate was the smallest component of dried larval tissues. Mean carbohydrate content decreased by about 80% between Day 1 (13.5 ± 4.9 ng $larva^{-1}$) and Day 4 (2.7 ± 0.8 ng $larva^{-1}$). Subsequently, carbohydrate content increased to 63.1 ± 31.1 ng $larva^{-1}$ on Day 21.

Lipid loss between Day 1 and Day 4 contributed 56% of the total energy utilised during this period, whereas protein contributed almost 40%. The energy contributed by carbohydrate was very small, less than 10% of the amount contributed by lipid. Between Day 18 and Day 21, the accumulation of lipid contributed almost 70% of the total energy gain per larva during this period. The energy available from lipid was more than four times that available from protein and carbohydrate, each of which contributed about 15% of the total energy value at this time.

Patterns of energy reserve composition, utilisation and accumulation within *P. margaritifera* larvae were found to be comparable to those reported for temperate species (Holland and Spencer 1973; Gallager *et al.* 1986; Whyte *et al.* 1987). As reported in all previous studies with bivalves (Holland 1978; Whyte *et al.* 1989), protein was the largest organic component of *P. margaritifera* larvae and showed the largest increase during larval development. Protein forms the bulk of the structural organic components of larval tissues (Holland 1978; Whyte *et al.* 1989). Also in accordance with previous studies, lipid was the second largest organic component of *P. margaritifera* larvae and carbohydrate content was relatively small and changed little during larval development.

The marked decline in protein, lipid and carbohydrate content of *P. margaritifera* larvae between Days 1 and 4 is similar to that reported for the larvae of temperate scallops (*Patinopecten yessoensis* and *Crassadoma gigantea*), where protein, lipid and carbohydrate were reported to be catabolised simultaneously and linearly with time during embryonic

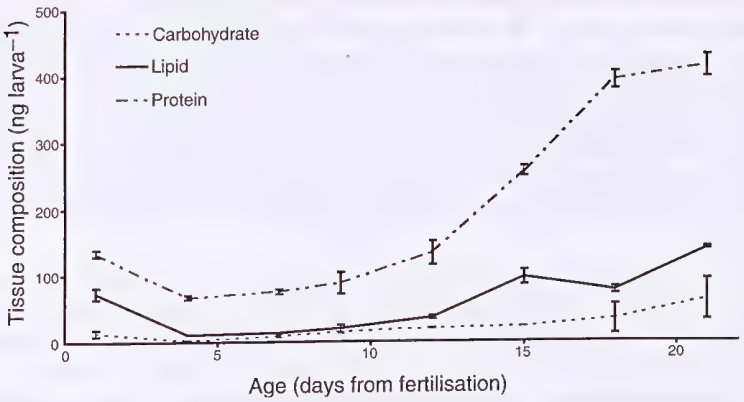

Fig. 1. Changes in mean (± SEM) protein, lipid and carbohydrate content of *P. margaritifera* larvae (ng larva^{-1}) during development ($n = 6$, Days 1 to 9; $n = 3$, Days 12 to 21).

development (Whyte *et al.* 1990, 1991). This decline is thought to result from utilisation of endogenous reserves, provided to the egg by the female parent, to fuel the transition to an exogenous mode of feeding (Bayne *et al.* 1975; Mann and Gallager 1985). Utilisation of protein, lipid and carbohydrate by *P. margaritifera* between Days 1 and 4 shows that larval development during this period is an energetically expensive process; approximately 66% of the total energy content of 1-day-old larvae was utilised by Day 4. Similarly, 56.9% of the energy content of fertilised eggs was reported to be utilised during the first 72 h after fertilisation in scallop (*Crassodoma gigantea*) larvae (Whyte *et al.* 1990).

Over half the energy utilised by *P. margaritifera* larvae between Days 1 and 4 was contributed by lipid, suggesting that lipid is the primary energy reserve during this period. Similarly, embryogenesis of the oyster (*Crassostrea virginica*) and clam (*Mercenaria mercenaria*) has been reported to be fuelled primarily (55–96% and 50–65% respectively) by parentally derived lipid (Gallager and Mann 1986).

A number of studies with temperate bivalves only investigated changes in the lipid content of tissues during larval development and disregarded changes in protein or carbohydrate contents (Gallager and Mann 1986; Gallager *et al.* 1986; Napolitano *et al.* 1988; Delaunay *et al.* 1992, 1993). However, in *P. margaritifera* larvae, protein was found to contribute 40% of the total energy utilised between Days 1 and 4, indicating it to be an important secondary energy source during this period. Similarly, Whyte *et al.* (1990, 1991) reported protein to contribute a significant portion of the total energy expended during the embryogenesis of the scallops, *Patinopecten yessoensis* and *Crassodoma gigantea* (44.9% and 43.5% respectively). Such values were only slightly lower than the energy contribution reported for lipid of 47.6% and 4% respectively (Whyte *et al.* 1990, 1991).

Although the overall energy contribution from carbohydrate in *P. margaritifera* tissues was small between Days 1 and 4, it accounted for an 80% depletion of its initial energy content, which is comparable to the decline in lipid (86%). Similar depletion of carbohydrate has been reported during embryogenesis of scallops (Whyte *et al.* 1990, 1991). In comparison, the decline in protein content of *P. margaritifera* larvae between Days 1 and 4 accounted for only 50% of the initial energy content. This reflects the important structural role of proteins in keeping the larval body intact (Holland 1978).

Larval lipid and carbohydrate contents increased markedly between Days 18 and 21. During the same period, the rate of protein accumulation declined considerably. Although both lipid and carbohydrate contents increased by almost 50% between Days 18 and 21, accumulation of lipid contributed more than four times the total energy gain (per larva) than either protein or carbohydrate. This strongly suggests that lipid may be the primary energy reserve utilised during metamorphosis. Although this assumption is supported by the results of similar studies with temperate species (Holland and Spencer 1973; Whyte *et al.* 1987), further study is required to confirm this role for lipid in *P. margaritifera*.

These results provide a strong basis for future study by clearly indicating changes in proximate composition and energy-reserve utilisation and accumulation during larval development of *P. margaritifera* larvae. This research has increased our understanding of the energetics of bivalve larvae, being the first study of its kind to investigate a tropical species. These findings have significant practical application and will be useful in the development of more efficient hatchery techniques for pearl oysters; for example, in formulating diets that best provide the biochemical requirements of larvae to maximise growth and survival and to minimise the time to metamorphosis.

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